

**INORGANIC ARSENIC EXPOSURE AND SEX-DEPENDENT SUSCEPTIBILITY TO
ISCHEMIC HEART INJURY**

by

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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the
degree of Master of Science

Baltimore, Maryland

April, 2018

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Abstract

Background: Epidemiological evidence suggests an association between cardiovascular disease and environmental pollutants. Arsenic is a common contaminant in drinking water throughout the world, and recent studies suggest a link between inorganic arsenic (iAS) exposure and ischemic heart disease. Although female hearts exhibit an estrogen-dependent reduction in susceptibility to ischemic injury compared to males, females may be especially susceptible to iAS due to endocrine disrupting effects. However, iAS exposure and susceptibility to ischemic heart injury have not been examined in mechanistic studies.

Methods: Male and female C57BL/6J mice (aged 8 weeks) were exposed to varying concentrations of sodium arsenite (0 parts per billion (ppb), 10ppb, 100ppb, 1000ppb) via drinking water for 4 weeks. Hearts were then excised and subjected to ischemia-reperfusion (I/R) injury via Langendorff perfusion. Pre and post-exposure echocardiography was also conducted, and post-exposure plasma samples were collected for 17 β -estradiol measurement.

Results: Mice exposed to 10ppb or 100ppb of iAs revealed no significant changes in gross cardiac morphology, functioning, or susceptibility to ischemia-reperfusion injury. At 1000ppb of iAS, female hearts exhibited modest structural remodeling with no change in heart function, while male hearts showed no change in cardiac morphology or function. Interestingly, we identified substantial sex-dependent changes in I/R susceptibility. iAS-treated female hearts showed a significant decrease in post-ischemic functional recovery and increased infarct size, while iAS-treated males showed significantly enhanced post-ischemic functional recovery and reduced infarct size. Assessment of plasma 17 β -estradiol levels revealed a decrease in iAS-treated females (vs. non-treated females), but this decrease was not significant. eNOS protein

levels were significantly decreased in whole heart homogenates from both iAS-treated male and female hearts. eNOS phosphorylation at Ser1177 was also significantly elevated in iAS-treated male hearts.

Conclusions: These results suggest that an environmental exposure such as iAS, can modulate susceptibility to ischemic heart disease. Our results further suggest that iAS can modulate myocardial nitric oxide signaling to either increase or decrease susceptibility to ischemic injury.

Acknowledgements

I would like to thank my advisor Dr. Mark Kohr for his guidance and support. Mark has been an excellent mentor and has helped me launch a career in scientific research. I would also like to thank all current members of the Kohr Laboratory of Cardiovascular Redox Signaling for their help and support. My thesis advisory committee members, Dr. Mark Kohr, Dr. Winnie Tang, and Dr. Charles Steenbergen provided guidance and were essential in the completion of my thesis. I would like to thank my family for their love, encouragement, and support throughout my time in graduate school.

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CHAPTER 1

Introduction and Background

1.1 General introduction

The cardiovascular system consists of the heart and a highly organized network of blood vessels, including arteries, veins, and capillaries, that serve as the blood circulation network of the body. The highly muscular heart generates the force necessary for the movement of blood throughout the body. The human heart consists of four chambers, including the right atrium, right ventricle, left atrium, and left ventricle. The right and left atria receive blood from veins whereas the left and right ventricle pump blood out of the heart through arteries. The heart pump has two sets of valves that maintain fluid flow in the correct direction. The two atrioventricular valves separate the atria from the ventricles. Two semilunar valves are located at the base of the major vessels joined to the ventricles. Separating the right atria and ventricle is the tricuspid valve, while the bicuspid valve (or mitral valve) separates the left atria and ventricle. The pulmonic valve separates the pulmonary trunk from the right ventricle, while the aortic valve separates the aortic arch from the left ventricle. When the ventricles contract, termed systole, the atrioventricular valves close to prevent backflow of blood from the ventricles into the atria. When the ventricles relax, termed diastole, the semilunar valves close to prevent backflow of blood from the aortic arch and the pulmonary trunk into the ventricles. The cardiovascular system's role of maintaining homeostasis relies on the coordinated function of the vessels, atria, and valves and the continuous movement of blood throughout the body in the correct direction. The circulating blood serves as the delivery system for essential materials as well as the mediator of cellular waste removal. Oxygen rich blood, required for cellular respiration, is pumped from the heart to the organs, penetrating the tissue through an integrative network of capillaries. Simultaneously, the blood

receives carbon dioxide and other waste products from cells where it can be transported to the kidneys and lungs and excreted from the body (Weinhaus and Roberts 2009).

Numerous mechanisms underlie the functions of the cardiovascular system. Any endogenous or exogenous agent that disrupts the normal structure or function of the heart or the peripheral vessels causes cardiovascular disease. The traditionally thought of risk factors for developing cardiovascular disease are generally modifiable behaviors, such as smoking and physical inactivity. Environmental exposures are now being recognized as a major risk factor for the development of cardiovascular disease. In contrast to smoking and physical inactivity, individuals often have little control over the exposures they face during their lifetime. The heart and vasculature network is sensitive to a variety of environmental agents, including pesticides, metals, and particulate matter. This thesis focuses on the effects of inorganic arsenic on the cardiovascular system. Inorganic arsenic is listed by the World Health Organization as one of the top 10 environmental contaminants of particular concern (Cosselman et al. 2015). The metalloid can be found at high concentrations in contaminated ground water. Chronic exposure to inorganic arsenic is linked to various fatal and non-fatal cardiovascular diseases, including atherosclerosis, hypertension, and ischemic heart disease. The mechanism(s) by which inorganic arsenic drives cardiovascular disease incidence has yet to be fully understood. This chapter will introduce a number of concepts, including basic cardiovascular physiology and pathophysiology, the phenomenon of sex-dependent cardioprotection with a focus on the role of estrogen signaling, and arsenic cardiotoxicity according to the previously published literature.

1.2 Myocardial physiology

The heart has an extensive role in maintaining homeostasis in the human body. A functional heart supplies blood to various tissues and organs that are undergoing metabolic processes with a high

demand for oxygenated blood that is rich in nutrients, metabolites, and hormones. Maintaining homeostasis in the human body requires the heart muscle, which is composed of cardiomyocytes or contractile cells, to contract and relax in a synchronized and sustained manner. The coordinated contraction and relaxation of the myocardium is referred to as the cardiac cycle and is driven by the action potentials initiated spontaneously by the autorhythmic fibers in the sinoatrial (SA) node, which is located in the wall of the right atrium. The heart muscle is unique in that the generation of the action potential does not require the input of the nerve stimuli like smooth or skeletal muscle. Instead, the SA node independently maintains the synchronized rhythm of the heart. While heart rate can be influenced by the sympathetic and parasympathetic nervous system, the SA node alone can maintain the coordinated contraction and relaxation of the heart muscle. The SA node also has the ability to alter the generation of action potentials to adapt to the changing demands of the body (Fishbein et al. 1981; Nabeebaccuus and Shah 2014).

1.2.1 Cardiac cycle

The cardiac cycle is initiated by the depolarization of the SA node. Cells of the SA node have a spontaneous gradual change in membrane potential which is referred to as diastolic depolarization (Li et al. 2016). The spontaneous diastolic depolarization of the SA node leads to the generation of an action potential referred to as the pacemaker potential. The mechanism by which the SA node cells spontaneously depolarize is still not fully understood. The classic mechanism of spontaneous depolarization is through the opening of hyperpolarization activated cyclic nucleotide (HCN) gated channels of the sarcolemma membrane, the opening of which allows for a mixed flux of sodium and potassium ions creating an inward current that is referred to as a funny current. The funny current allows the SA node cells to depolarize and to reach the threshold that allows voltage gated sodium channels to open (Joung et al. 2009). Once the action potential is generated by the pacemaker cells it can travel throughout both atrial chambers via the

gap junctions of the intercalated discs, located between the tightly packed myocardial fibers to allow for the contraction of both atria simultaneously. The action potential reaches the atrioventricular (AV) node and is slowed down to allow for both atria to fully contract and empty into the ventricles through the tricuspid and mitral valves. At this point, the action potential has not reached the ventricles, as the chambers are insulated by a surrounding fibrous skeleton. The action potential spreads from the AV node through the bundle of His, where it propagates through the septum by means of the right and left bundle branches. When the action potential reaches the apex of the heart, the Purkinje fibers rapidly conduct the action potential upward causing the ventricles to contract (Wilcken 2012). This allows for blood to be pushed up and through the aortic and pulmonary valves overcoming the afterload or outflow resistance of the vasculature.

1.2.2 Cardiac contractile machinery

The heart is comprised of cardiomyocytes that make up the majority of the heart's mass. Unlike skeletal muscle fibers, cardiac muscle fibers are branched and shorter in length. Cardiac muscle has a high number of mitochondria that are much larger than those of the skeletal muscle. This allows the cardiac muscle cells to meet the high energy demands of the continuous energy dependent contractile processes. Cardiomyocytes are tightly packed together by desmosomes and connected by gap junctions that allow for the rapid spread of action potentials and the synchronized contraction of the muscle. Because nutrients are unable to diffuse rapidly into the cells that make up the cardiac walls as the chambers of the heart are being perfused, the myocardium requires its own network of blood vessels. Two coronary arteries, the right and left coronaries, descend in a branching manner from the aorta towards the apex of the heart to supply oxygenated blood to the entire myocardium. The majority of the coronary blood supply is to the left ventricle and this is greatest during ventricular diastole (Nabebaccuus and Shah 2014). Cardiomyocytes have a resting membrane potential of roughly -90 mV due to the leaching of

potassium cations through inward-rectifier potassium ion channels of the sarcolemma membrane. An action potential, initiated by the pacemaker cells of the SA node, will spread along the sarcolemma and T-tubule network of a myocyte allowing the membrane potential to reach threshold at roughly -70 mV initiating the opening of voltage gated fast sodium channels. This allows for the rapid influx of sodium down the electrochemical gradient of the interstitial fluid and the cytosol of the cardiomyocyte. This inflow of sodium ions results in the rapid depolarization of the cell membrane to slightly above 0 mV causing the sodium channels to close (Nabeebaccuus and Shah 2014). During this depolarization, voltage gated L-type calcium channels open and a steady influx of calcium occurs down the concentration gradient into the cytosol of the myocytes. This inflow of calcium cations stimulates additional calcium ions to be released from the sarcoplasmic reticulum, which is the major calcium store in the cardiomyocyte, into the cytosol through the sarcoplasmic reticulum calcium release channel (or ryanodine receptor, RyR) (Wang et al. 2008). This calcium-induced calcium release is required for muscle contraction, as the initial calcium influx is insufficient to meet contractile requirements. The contractile machinery is stimulated by the binding of calcium to troponin-C (Winslow et al. 2016). The proteins of the troponin complex consist of three globular subunits that allow for its regulatory function. The troponin complex simultaneously binds to actin, couples to adjacent tropomyosin strands that conceal myosin binding sites on actin, and presents a binding site for calcium. The sarcomere contractile unit contains thick filaments composed of the protein myosin and thin filaments composed of actin. The binding of calcium to troponin drives a conformational change in the troponin-tropomyosin complex to reveal myosin binding sites on the thin filaments of actin. Muscle contraction is initiated by the binding of ATP to myosin. ATP is hydrolyzed to a phosphate ion and ADP and cross bridge formation occurs between actin and the myosin head. The accumulation of cross bridges drives a power stroke, characterized by the shortening of the sarcomere in which the thin and thick filaments have an increased region of overlap. This state of the sarcomere is maintained until another ATP binds to the myosin head. The above description

of muscle contraction is referred to as the sliding filament theory of muscle contraction. The theory was developed by Hugh Huxley and Jean Hanson in 1954 and is still universally accepted as the mechanism of muscle contraction (Huxley and Niedergerke 1954; Huxley and Hanson 1954). The period of time that contraction occurs is referred to as the plateau phase. During this time, the influx of calcium ions is balanced with an outflow of potassium ions maintaining a stable depolarized electrical potential. After contraction, calcium dissociates from troponin-C and repolarization of the myocyte occurs through the outflow of potassium ions through various potassium ion channels of the sarcolemma. Relaxation of the muscle occurs as calcium is extruded from the cell or re-sequestered into the sarcoplasmic reticulum and the potassium efflux restores the myocytes electrical potential to the resting potential of -90 mV. Maintaining steady state calcium homeostasis is critical for proper myocyte relaxation. Steady state calcium homeostasis is only maintained when the amount of calcium entering the cell along with contraction through L-type channels equals the amount of calcium extruded from the cell before the subsequent contraction. The resting calcium gradient across the sarcolemma and the available intracellular calcium store of the sarcoplasmic reticulum directly correlates with the force of contraction of the myocyte. Chemical agents that alter calcium homeostasis also alter the force of contraction of the myocyte and thus the force generated by the heart (Barry and Bridge 1993). After restoring calcium homeostasis, sarcolemma repolarization, and the completion of the absolute refractory period the cardiomyocyte can respond to another action potential propagating through the heart (Nabeebaccuus and Shah 2014).

1.3 Cardiovascular pathology

Cardiovascular disease refers to the diseases of the heart and blood vessels. This includes but is not limited to congestive heart failure, atrial fibrillation, high blood pressure, stroke, and ischemic heart disease, also referred to as coronary heart disease. Of all the cardiovascular diseases,

ischemic heart disease is the most common cause of death worldwide claiming an estimated 8.91 million lives in 2015 according to the Global Burden of Disease Study (Roth et al. 2017). In the early 2000s advancements in biomedical research and technology allowed for great strides in the effective treatment of heart disease. Heart related mortality rates declined by an average of 3.7 percent per year from the year 2000 to 2011. In the last few years this trend has reversed as the risk factors for heart disease in the American population have rapidly increased. The American Heart Association reports that in the year 2015 the mortality rate for heart disease increased by 1 percent for the first time since the year 1969. A 2011 review from the American Heart Association predicted that roughly 40 percent of the American population would suffer from cardiovascular disease by 2030. The organization has now recalculated the projection as the early estimate was surpassed in 2015 with a cardiovascular disease prevalence of 41.5 percent of the American population. The American Heart Association now estimates that 45 percent of the American population will suffer from cardiovascular disease by 2035 (Benjamin et al. 2017).

1.3.1 Environmental determinants of cardiovascular disease

Despite national efforts to improve cardiovascular health, the prevalence and control of cardiovascular risk factors in the United States remains a major issue. The persistent growth in the risks of cardiovascular disease exemplifies our lack of a complete understanding of the underlying risk factors contributing to the growing burden of heart disease hindering effective prevention and treatment (Benjamin et al. 2017). Existing research suggests that the majority of cardiovascular disease is preventable highlighting the environmentally driven nature of heart disease. Less well understood is how environmental risk factors influence heart disease. Rates of cardiovascular disease differ drastically among different populations of different geographical regions (Willett 2002). The mortality rates for cardiovascular disease are roughly 7 to 14 times higher in Northern and Eastern Europe when compared to other countries including Japan and

Argentina (Levi et al. 2002). Migrant studies indicate that when groups move from low to high risk countries, their disease rates begin to resemble the populations of the new environment (Bhatnagar et al. 1995; Patel et al. 2006; Worth et al. 1975). Epidemiological analysis suggests that changes in the environment and life style factors such as nutrition and exercise could decrease the incidence of coronary artery disease by roughly 80 percent and stroke incidence by 70 percent (Willett 2002). Generating a complete understanding of cardiovascular disease therefore requires an approach that utilizes the disciplines of the environmental sciences and cardiology. Numerous epidemiological studies have provided direct links between heart disease and exposure to common environmental pollutants like particulate matter, metals, and toxic gases but the extent at which these pollutants contribute to the manifestation of the diseases remains unclear. The toxicopathological mechanisms by which environmental pollutants drive different forms of heart disease have yet to be fully identified (O'Toole et al. 2008). An expansion upon our knowledge on the underlying mechanism by which environmental pollutant exposures drive the manifestation of cardiovascular disease is essential for the development of effective treatment options and population prevention techniques for heart related diseases.

1.3.2 Ischemia-reperfusion injury

Myocardial ischemia, the imbalance of cardiac blood supply and demand, is the main clinical symptom of ischemic heart disease. Myocardial infarction results from periods of myocardial ischemia driven primarily by the occlusion of the coronary arteries that sit on the epicardial surface of the heart. Myocardial ischemia occurs when the blood flow to the heart is reduced or lost and the myocardium is subjected to a prolonged period of anoxia and nutrient deprivation. The heart requires an immense amount of oxygen to meet the aerobic metabolic needs of the mitochondria in cardiomyocytes. The electron transport chain is hindered by the lack of oxygen, leading to a drastic decline in ATP levels and the disruption of cytoplasmic ion concentrations as

the sarcolemmal Na^+/K^+ ATPase and the sarcoplasmic reticulum Ca^{2+} ATPase can no longer mitigate active transport of ions. This results in elevated levels of cytosolic calcium and sodium in the myocyte and dysfunction in the calcium driven contractile machinery. The diminishment of oxidative phosphorylation drives an increase in anaerobic respiration and an accumulation of lactic acid. Proton concentrations rise rapidly, lowering the intracellular pH and inhibiting the glycolytic enzyme activity further exacerbating the diminished levels of ATP. High proton concentrations also drive the cellular import of sodium ions through the Na^+/H^+ Transporter of the sarcolemma. To compensate for high sodium levels, the cell reverses the activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger resulting in a further overload of cytosolic calcium levels. Elevated levels of cytosolic calcium disrupts the normal synchronized pattern of myocyte contraction. Additionally, calcium overload initiates various non-caspase proteases that are implicated in apoptosis and necrosis including calpain and cathepsins. Without timely reperfusion the profound cellular dysfunction results in irreversible myocardial infarction and a loss in overall heart function. While reperfusion therapies are necessary to restore blood flow to the ischemic myocardium to retrieve functional heart tissue, the intervention results in additional cardiac injury due to the many complications associated with the abrupt reperfusion of the coronary artery flow. During ischemia moderate levels of superoxide dismutase are produced in response to the oxidative stress. With the onset of reperfusion there is a burst of mitochondrial derived reactive oxygen species (ROS) production and the saturated superoxide dismutase is unable to compensate. Elevated cellular ROS levels, accompanied by calcium overload, leads to mitochondrial induction of apoptotic and necrotic cellular pathways. ROS, calcium overload, ionic imbalance, reduced ATP production, and lowered pH all appear to be factors implicated in the cardiomyocyte death associated with the Ischemia-reperfusion injury. Ischemia-reperfusion injury has been observed to be extremely time sensitive. The extent of injury is directly proportional to the length of the ischemic period. While 5 minutes of ischemia can result in slight cardiac arrhythmias, periods of ischemia greater than 20 minutes can lead to irreversible necrosis or apoptosis (Powers et al.

2007). The infarcted heart tissue lacks contractility and prevents the heart from appropriately functioning as the blood pump of the body.

1.3.3 Cardiac remodeling

Cardiac remodeling is either physiological or pathological and describes the changes in size, shape or function of the myocardium that results from internal or external stimuli that cause biological or mechanical stress. Cardiac remodeling can be either a beneficial result of regular exercise or a maladaptive response to various chronic stressors, including pressure overload, volume overload, or myocardial infarction. Increased fluid volume or pressure incident on the heart chambers results in a stretching force on the myocardium. Stretching caused by the increased work load results in a hypertrophic response, or growth in size of the cardiomyocyte. In response to normal physiological stress, cardiomyocytes respond with the synthesis of new contractile proteins and the formation of new sarcomeres aligned in a highly organized parallel manner. This type of remodeling, termed physiological hypertrophy, is reversible and allows for the generation of greater force with each contraction. In response to chronic stress, irreversible remodeling occurs. Pathological hypertrophy is characterized by the elevated and disproportionate contribution of cardiac fibroblasts, which produce excessive amounts of collagen leading to fibrosis and the stiffening of the myocardium. Myocytes are eventually replaced with more fibroblasts and extracellular collagen. With prolonged pathological cardiac remodeling, the heart loses the ability to undergo hypertrophy and to adapt to changing fluid volumes and pressures (Frey and Olson 2003; Muhl et al. 2008). High blood pressure is considered the most important risk factor for cardiac remodeling and heart failure. The cardiac hypertrophy of hypertensive patients is a response to the afterload that results from the stiffening of the vessels. In the case of ischemic injury and myocardial infarction, cardiomyocytes must grow in size to assume the work of the heart segment that was lost (Burchfield et al. 2013).

1.3.4 ROS and oxidative stress

When examining cardiac injury as it relates to an environmental stressor like arsenic exposure, it is important to consider the effects of ROS. ROS includes superoxide, hydrogen peroxide, and the highly reactive hydroxyl radical. It is well known that an increase in ROS is directly associated with ischemia-reperfusion injury. ROS are generated from multiple sources including the mitochondrial electron transport chain, xanthine oxidase, and NADPH oxidase (phagocyte oxidase) (Figure 1). The mitochondrial generation of ROS is recognized as the primary contributor to ischemia-reperfusion related oxidative stress. It is estimated that roughly 95% of oxygen is reduced to water through the mitochondrial electron transport chain. The remaining 5% of oxygen cycling through the electron transport chain generates the highly reactive superoxide anion. Xanthine oxidase is a mammalian redox enzyme with relatively low specificity as it catalyzes the reduction of a number of species including oxygen, cytochrome c, and nitric oxide (Pacher et al. 2006). The NADPH oxidase is a neutrophil enzyme that catalyzes the production of superoxide from molecular oxygen (Segal 2008). At low levels, ROS may have protective roles via signaling for preconditioning or through oxidant induced gene transcription. Under normal conditions, our tissues have a specialized system which is sufficient for keeping ROS levels low, thereby preventing extensive oxidative damage by an overabundance of superoxide radicals and other ROS. Superoxide dismutase (SOD) reduces superoxide to the less reactive hydrogen peroxide. The hydrogen peroxide is further reduced to water by the enzyme catalase or through the glutathione peroxidase system (Figure 1) (Becker 2004). Transgenic mice overexpressing manganese superoxide dismutase (SOD2), the SOD enzyme isoform restricted to the mitochondrial matrix and functioning to reduce ROS levels within the mitochondria, were observed to be protected against myocardial ischemia-reperfusion injury. This provides substantial evidence that the oxidative stress implicated in the injury is largely due to

mitochondrial derived ROS rather than from exogenous sources of ROS (Powers et al. 2007). When oxidant scavenging systems are saturated and hydrogen peroxide concentrations are elevated within the cell, hydrogen peroxide can react with metal ions via the Fenton reaction to form the highly reactive and destructive hydroxyl radical. Hydroxyl radicals readily oxidize lipids, DNA, and proteins (Figure 1) (Becker 2004). Considering the role of the mitochondria in ROS generation is vital to understanding ischemia-reperfusion injury.

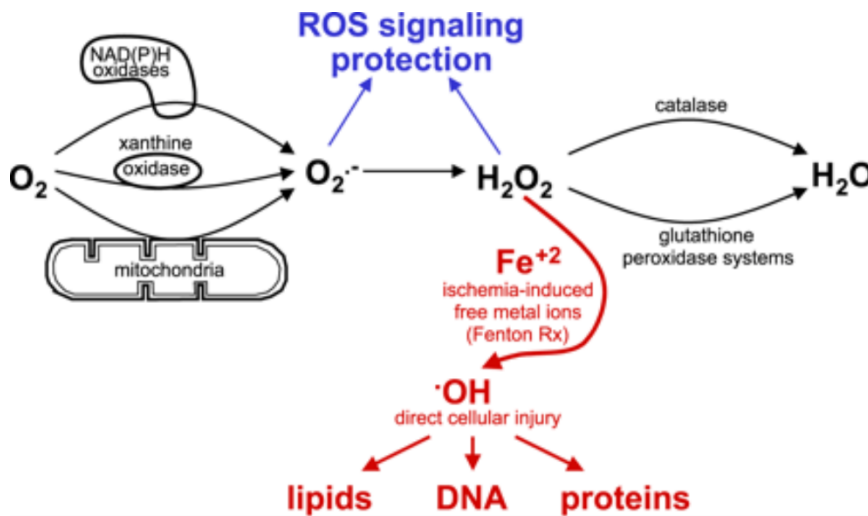


Figure 1. ROS production and metabolism (Becker 2004).

1.3.5 Mitochondrial ROS generation

The mitochondria is the driver of metabolism within the cell, generating the majority of the ATP necessary for a variety of cellular processes in the myocyte, including active transport mechanisms, as well as the coordinated contraction and relaxation of the cardiomyocyte machinery. Mitochondria play a central role in ROS related molecular pathology. During ischemia, the mitochondria becomes compromised due to the stressful hypoxic conditions which include calcium overload, an acidic pH, disrupted ion concentrations, and rapid ATP depletion.

When the ischemic tissue is reperfused, damaged mitochondria leak electrons from a number of locations on to molecular oxygen to form superoxide. This description of mitochondrial ROS production is an overly simplified explanation that does not encompass the highly complex nature of ROS generation. The true mechanism of mitochondrial ROS production has not been fully developed. However, Complex I and complex III of the electron transport chain have been identified as the major sites of ROS generation under stressful conditions (Murphy 2016).

1.3.6 Cardiomyocyte death

Mitochondria can become damaged by endogenous and exogenous ROS, leading to necrosis and apoptosis, which occurs through a variety of cellular pathways. Elevated ROS levels in the cell, accompanied by calcium overload, can lead to an increase in the inner or outer mitochondrial membrane permeability and the induction of apoptotic and necrotic cellular pathways. The two pathways leading to cellular apoptosis are called the extrinsic and intrinsic pathways. The extrinsic pathway occurs in the cytosol through the ligation of death receptors that drive the formation of the Fas-associating death domain-containing protein (FADD) oligomer located within the death inducing signaling complex (DISC). FADD causes the dimerization and activation of the initiator caspases 8 and 10. The initiator caspases cleave and activate the effector caspases of the proteasome and thereby trigger apoptotic cell death through the cleavage of specific substrates in the cell (Parrish et al. 2013). The intrinsic pathway is driven by the mitochondria and is the pathway implicated in ischemia-reperfusion injury. The combination of ischemia and reperfusion induces the translocation of pro apoptotic proteins of the Bcl-2 family from the cytosol to the outer membrane of the mitochondria. The Bcl-2 proteins bind to the outer membrane and increase the permeability of the membrane, which allows for the release of apoptotic proteins from the inner membrane space. The primary apoptotic protein implicated in the intrinsic cascade of cellular apoptosis is cytochrome c. Cytochrome c and the presence of

ATP allows for the activation of the adapter molecule apoptosis protease activating factor 1 (APAF-1) within the apoptosome multiprotein complex. The apoptosome can then activate the effector caspases that trigger apoptotic cell death through the cleavage of specific substrates in the cell. In addition to the release of cytochrome c, Smac/DIABLO, htrA2/omi protease, and Endonuclease G are released from the mitochondrial inner membrane space. The Smac/DIABLO and htrA2/omi proteins activate the effector caspases through the degradation of caspase inhibitory proteins. Endonuclease G is responsible for DNA fragmentation in the nucleus (Baines 2009). The apoptotic proteins collectively drive the death of the cardiomyocyte. Cardiomyocyte death is particularly problematic in mammalian species due to the lack of proliferation of adult cardiomyocytes. Cardiomyocytes rapidly proliferate during fetal life but lose this ability soon after birth with the withdrawal from the cell cycle. Cardiomyocytes are referred to as terminally differentiated because the cells are generally unable to reenter the cell cycle. Although the extent to which the adult mammalian cardiomyocyte is able to reenter the cell cycle is debated, most cardiac growth occurs through hypertrophy (Ahuja et al. 2007; Senyo et al. 2014).

1.3.6.1 Mitochondrial permeability transition

In addition to the intrinsic and extrinsic pathways of apoptosis, both cellular necrosis and apoptosis are believed to be driven by the induction of the mitochondrial permeability transition (MPT). Excessive ROS and calcium overload in the mitochondrial matrix associated with reperfusion leads to the opening of a large inner membrane pore called the mitochondrial permeability transition pore (MPTP). The opening of the pore causes the loss of the proton electrochemical gradient across the inner membrane, disrupting ATP generation and inducing additional ROS generation. With the opening of the pore, selectivity across the inner membrane is lost. The mitochondrial stress can lead to the mitochondrial matrix swelling and the rupture of the

organelle, the release of apoptotic proteins, and cellular apoptosis by the intrinsic pathway.

Because apoptosis requires the presence of ATP, severe ATP depletion from the mitochondrial permeability transition can instead result in cellular necrosis. The balance between ATP depletion from the MPT and the generation of ATP from glycolysis is what determines whether the cell death will result from apoptosis or necrosis (Kim et al. 2003). Due to the MPTP's central role in both apoptosis and necrosis, the MPTP is believed to be an important target for cardioprotection. Pre-conditioning with specific MPTP inhibitors has been shown in both animal and clinical studies to reduce ischemia-reperfusion injury (Ong et al. 2015).

1.3.7 Cardioprotection

With myocardial ischemia-reperfusion injury being a major cause of morbidity and mortality in the world, cardioprotective strategies have great potential for reducing a significant health care burden (Schwartz Longacre et al. 2011). The prognosis after a myocardial ischemic injury is primarily dependent on myocardial infarct size. Cardioprotection refers to the strategies that are employed to limit myocardial infarct generation resulting from both the ischemic event and the related reperfusion injury. Cardioprotection can be achieved through a variety of interventions ranging from ischemic preconditioning, the treatment with multiple periods of brief ischemia, to the usage of pharmacological agents. Acute cardioprotective effects are believed to be generated through complex cascades of cellular signaling pathways that dictate protein expression and activity. There are three potential time points for a clinical intervention during an ischemic event referred to as pre, per, and post conditioning. Preconditioning refers to the cardioprotection strategies that are implemented before an ischemic event, while perconditioning refers to the strategies employed during an ischemic event. Lastly, postconditioning refers to the strategies employed after the ischemic event and at the onset of reperfusion (Heusch 2015). Preconditioning with multiple periods of brief ischemia remains as one of the most powerful methods for reducing

myocardial infarct size. Ischemic preconditioning is however impractical for addressing an unpredictable acute injury in the clinical setting. There is a need to develop pharmacological strategies that mimic the beneficial effects gained from ischemic preconditioning (Schwartz Longacre et al. 2011). Our group and others have demonstrated that there is a significant increase in a cysteine-based post translational modification, S-nitrosylation (SNO), with multiple models of cardioprotection (Kohr et al. 2011a; Kohr et al. 2011b; Kohr et al. 2012; Kohr et al. 2014; Lochner et al. 2000; Shao et al. 2016; J Sun et al. 2006). The cysteine-based post-translational modification of SNO is a specific and reversible modification formed through the covalent attachment of nitric oxide (NO) to thiol groups. NO holds promise as a critical component of cardioprotection through the cellular signaling mechanism of SNO.

1.3.8 Nitric oxide as a cardioprotective agent

NO is a gaseous free radical signaling molecule that is heavily implicated in cellular signaling processes within the cardiovascular system. Of the RNS, NO is the most reactive species. NO is involved in both autocrine and paracrine cellular signaling, being able to diffuse across cellular membranes of all cell types. Since the identification of NO as the endothelium derived relaxing factor (EDRF) and as a physiological regulator of blood pressure, NO has become one of the most important signaling molecules in the cardiovascular system. The discovery of NO provided a clear understanding of the mechanism of nitroglycerine, which has been administered for the treatment of angina symptoms since the beginning of the 20th century (Strijdom et al. 2009). Initially it was thought that NO could serve only as a regulator of blood pressure. After being validated as a cellular signaling molecule, NO has become recognized as being involved in numerous physiological processes including the immune response, transcriptional regulation, metabolism, apoptosis, and neural transmission (Forstermann and Sessa 2012)

1.3.8.1 Nitric oxide molecular targets and cellular signaling

NO interactions can be characterized by NO's four primary molecular targets. NO can interact with metals like iron and copper, forming metal-nitrosyl-complexes. NO's interaction with metals can have profound effects on enzyme activity as many biological enzyme active sites contain metal prosthetic groups that are necessary for carrying out catalytic functions (Cooper 1999; Mingos 2014). NO readily reacts with superoxide radicals to form a highly reactive peroxynitrite radical species. Peroxynitrite radical formation can lead to the nitration of the amino acid tyrosine (Moreira et al. 2016; Valez et al. 2012). This interaction is important when examining the nitroso-redox equilibrium, as tyrosine nitration can be a marker for high peroxynitrite levels in the cell and indicative of nitrosative stress (Peluffo and Radi 2007). NO reacts with thiols and in particular, the thiols of cysteine amino acid residues. Cysteine nitrosylation is an important post translational modification that regulates protein functions in the cell. The reversible nature of cysteine nitrosylation allows NO to act as a key signaling molecule (Martinez-Ruiz and Lamas 2007; JH Sun et al. 2006). Lastly, the NO free radical can react with oxygen or carbon dioxide to form the less reactive, but more stable nitrite (NO_2^-) and nitrate (NO_3^-) species. The reversible nature of this reaction allows nitrate and nitrite to be an important source of NO as both species can be reduced back to NO under specific physiological conditions (Lundberg et al. 2008). NO signaling occurs through two distinct mechanisms, cGMP dependent and cGMP independent. The role of NO was first discovered through the cGMP dependent mechanism which refers to the NO mediated activation of soluble guanylyl cyclase (sGC) in vascular smooth muscles cells. Activation of soluble guanylyl cyclase allows for the catalytic conversion of guanosine 5'-triphosphate (GTP) to cyclic guanosine 3',5'-monophosphate (cGMP) in the presence of a magnesium cofactor. After the initial discovery of NO signaling, the cGMP dependent mechanism was believed to be NO's sole mechanism of cellular signaling. NO has since been

recognized to have the ability to act as a cell signaling molecule independent of cGMP through the transient post-translational modification of SNO (Tuteja et al. 2004).

1.3.8.2 Sources of nitric oxide

NO is either produced by the enzyme nitric oxide synthase (NOS) from L-arginine and molecular oxygen or by NOS-independent mechanisms through the reduction of nitrites and nitrates. Three distinct isoforms of NOS function as the primary source of NO within the myocardium (Barouch et al. 2002). NOS is an oxidoreductase enzyme that functions to produce NO through the oxidative conversion of L-arginine to L-citrulline. The overall reaction consists of a two-step oxidative conversion of L-arginine to NO and L-citrulline with N^W-hydroxy-L-arginine as an intermediate and NADPH as an electron donating cofactor (Figure 2) (Aktan 2004). As a dimer consisting of two identical subunits, the enzyme properly functions to produce NO. Each subunit consists of two distinct domains, the C-terminal reductase domain and the N-terminal oxygenase domain. The C-terminal reductase domain contains binding sites for the nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and FMN reduction coenzymes necessary for full redox function. The N-Terminal oxygenase domain contains binding sites for heme, tetrahydrobiopterin (BH₄), and the L-arginine substrate. Located between both terminal domains is a calmodulin binding site that functions to regulate enzyme activity (Alderton et al. 2001). Three NOS isoforms exist of which two are constitutively expressed in the cell and one, as an element of the immune system, is induced during periods of stress including infection. The two constitutively expressed isoforms, endothelial NOS (eNOS) and neuronal NOS (nNOS), require calcium for activation. Intracellular calcium binds to calmodulin forming a complex which can then bind to eNOS or nNOS at the calmodulin binding site, inducing a conformational change that activates the NOS isoform. The other isoform, inducible NOS (iNOS), is present at high levels only when cytokines or other inflammatory agents induce

expression. The inducible isoform functions independent of intracellular calcium activation as calmodulin is always bound to the dimer with or without calcium present (Andrew and Mayer 1999). While Inducible NOS is only present under specific conditions, iNOS has the ability to produce much greater amounts of NO than the constitutively expressed isoforms of NOS (Aktan 2004). The NOS-independent mechanism of NO production is termed the nitrate-nitrite-NO pathway. The ability for nitrates and nitrites to be reduced to NO allows for an individual's diet to serve as a key source of NO. Diets suggested to be protective against cardiovascular disease, like the Mediterranean diet, are rich in inorganic nitrates. Leafy green vegetables contain particularly high levels of nitrates that can serve as substrates for NO production mechanisms (Capurso et al. 2014). Bacteria in the gut and oral cavity can convert nitrates to nitrites via the bacterial nitrate reductase enzymes (Tiso and Schechter 2015).

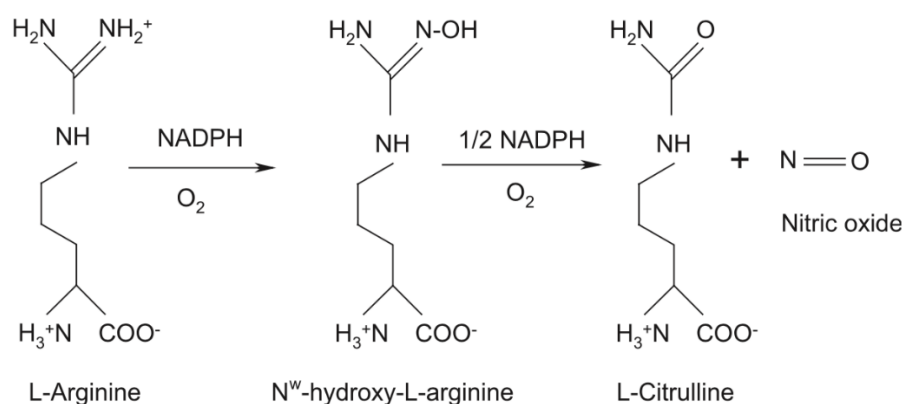


Figure 2. Nitric oxide synthase catalyzed conversion of L-arginine to L-citrulline and nitric oxide (Aktan 2004).

1.3.8.3 Nitroso-redox equilibrium and cardiovascular health

Within the cell, there is a balance between the production of ROS and the production of reactive nitrogen species (RNS) that is termed the nitroso-redox equilibrium, and this equilibrium appears

to be extremely important in maintaining cellular homeostasis. At moderate levels, both ROS and RNS are involved in cell signaling pathways. It is evident that the disruption of the nitroso-redox balance contributes to ischemia-reperfusion injury and many forms of cardiovascular disease. Cellular stress occurs at both high levels of ROS and high levels of RNS. Considering the interaction between ROS and RNS is crucial when examining an environmental pollutant's toxicophysiological effects on the cardiovascular system. Various forms of cardiac dysfunction have been linked to reduced levels of active NOS, which can be caused by a decrease in the transcriptional and translational expression of NOS or from the uncoupling of the NOS dimer. NOS uncoupling occurs most often when the cofactor BH₄ is depleted, which leads to the catalysis of superoxide production rather than NO production (Silberman et al. 2010). NOS can also be activated or inactivated through post-translational modification, including phosphorylation, acetylation, acylation, and s-nitrosylation (Qian and Fulton 2013; Sharma and Patel 2017). The upregulation of NO signaling and protein SNO is believed to be cardioprotective by altering the function and stability of proteins and by protecting thiol groups from the oxidative damage resulting from excessive ROS levels characteristic of ischemia-reperfusion injury (Kohr et al. 2011b; Kohr et al. 2014; Zweier and Talukder 2006). Excessive accumulation of SNO may exaggerate myocardial injury. There appears to be a threshold at which the protection from SNO is lost and nitrosative stress is triggered. In addition to the regulation of SNO levels by changes in expression and activation of NOS, SNO levels are regulated by S-nitrosoglutathione reductase (GSNOR) (Foster et al. 2012; Liu et al. 2004; Sips et al. 2013). NO can react with the antioxidant glutathione (GSH) to form S-nitrosoglutathione (GSNO). GSNO acts as a reservoir of NO in the cell and serves as a donor in the direct transfer of NO from the antioxidant to another protein referred to as trans-S-nitrosylation. GSNOR regulates SNO levels in the cell by catalyzing the conversion of GSNO to GSH (Liu et al. 2004). The maintenance of SNO levels within the cardiomyocyte appears to be a critical component for cardiac function. Females are believed to have an endogenous mechanism for the regulation of the nitroso-redox equilibrium during

ischemia-reperfusion injury. Understanding the cellular signaling basis of the female's innate resilience to ischemia-reperfusion injury will provide insights into how to better prevent irreversible myocardial infarct generation from an ischemic event.

1.4 Sex-dependent cardioprotection

Early epidemiological studies of cardiovascular disease focused on males, working under the assumption that the findings could be extrapolated to the management of heart disease in women. That assumption has since been proven to be incorrect as many longitudinal studies distinguishing women from men have illuminated the lower risk of cardiovascular injury for premenopausal women. The incidence of heart disease is shown to drastically increase with the onset of menopause (Hayward et al. 2000). It is evident that there are significant physiological differences between male and female hearts that contribute to the differences in the onset and presentation of cardiovascular disease. Because of these differences, the risk factors for coronary heart disease are weighted differently for women than men. At younger ages, smoking is much more detrimental on health status for women than men. By observing the age of onset of acute myocardial infarction compared to the individuals smoking habits, it has been observed that women lose approximately twice as many life years from smoking than men do (Grundtvig et al. 2009). While cardiovascular disease occurs much later in women than in men, an estimated 7 to 10 years later, heart disease remains the leading cause of death in women (Maas and Appelman 2010). Understanding the basis of this lower incidence of heart disease in females could provide insights into how to better treat and manage heart disease in both females and males.

A current hypothesis that is actively being investigated is that endocrine and paracrine estrogen signaling is at the root of the sex-dependent cardioprotection phenomenon (Hale et al. 1996; Hisamoto et al. 2001; Nikolic et al. 2007; MJ Wang et al. 2006). By assessing the reproductive

hormone levels of individuals being screened for heart disease, the NHLBI Women's Ischemia Syndrome Evaluation (WISE) study showed that premenopausal women experiencing coronary artery disease presented with significantly lower levels of estradiol and follicle stimulating hormone (FSH) than women without coronary artery disease (Bailey Merz et al. 2003). The cardioprotective role of estrogen is still heavily debated as hormone replacement trials for postmenopausal women have had negative outcomes. The large randomized Women's Health initiative controlled trial for estrogen-progesterone replacement therapy for postmenopausal women was stopped after roughly 6 years of follow up due to an observed increased risk of developing breast cancer which exceeded any cardioprotective benefits gained from the estrogen-progesterone treatment (Rossouw et al. 2002). A better understanding of estrogen mediated protection in animal models is required before a successful translation to a clinical therapy can be made as an adverse outcome of breast cancer cannot be ignored.

Sex-dependent protection from cardiovascular disease has been observed through numerous animal studies. Female animals are observed to have an improved post-ischemic functional recovery and smaller infarct size following ischemia-reperfusion injury when compared with males (Bae and Zhang 2005; Johnson et al. 2006). An abolishment of female cardioprotection to ischemia-reperfusion injury is observed in ovariectomized mice, further strengthening the theory of estrogen mediated cardioprotection (Ross and Howlett 2012).

1.4.1 The physiological role of estrogen and estrogen receptors

Estrogen is the female sex hormone that drives various physiological effects by binding to the estrogen receptors alpha and beta. The nuclear estrogen receptors act as ligand gated transcription factors. The nuclear estrogen receptors remain in transcriptionally inactive states in a complex with different nuclear chaperones when the estrogen ligand is not present. An estrogen receptor is

kept in its inactive state by various heat shock proteins. Upon binding of the estrogen ligand, the heat shock proteins dissociate from the receptor leading to the activation of the estrogen receptor (LeBlanc et al. 2009). Once activated, estrogen receptors have multiple modes of action. Ligand activated estrogen receptors can bind directly to estrogen response elements in DNA driving transcription of different genes. An estrogen receptor can also be activated independent of ligands via post translational modifications like phosphorylation. Activated estrogen receptors can facilitate protein-protein interactions of other transcription factors and indirectly affect regulation of genes that do not contain estrogen response elements. In addition to the transcriptional regulation of genes, estrogen and ligand activated estrogen receptors can drive cellular signaling through the activation of kinases and phosphatases, and by dictating ion fluxes across cellular membranes (Heldring et al. 2007).

1.4.2 Estrogen mediated cardioprotection

Several animal studies have shown estrogen and the activation of the estrogen receptors to be cardioprotective during ischemia-reperfusion injury (Booth et al. 2005; Hale et al. 1996; Lagranha et al. 2010; Lin et al. 2009a; M Wang et al. 2006). Estrogen is traditionally thought to be cardioprotective through the activation of estrogen receptors and the upregulation of proteins. In addition to the genomic effects, estrogen has been observed to drive rapid short term effects in the heart through the activation of various signaling pathways. Estrogen can illicit these rapid cardioprotective effects by binding to extra-nuclear estrogen receptors that are coupled to kinases. Both estrogen receptor- α (ER α) and estrogen receptor- β (ER β) have extra-nuclear actions involving the activation of kinases and subsequent acute signaling pathways (Menazza and Murphy 2016; Wu et al. 2011). Estrogen can also activate acute signaling cascades by binding to the transmembrane G-protein coupled receptor (GPR30) which is localized at the plasma membrane of the cell (Haas et al. 2007; Revankar et al. 2005). While it is recognized that

estrogen is cardioprotective in the setting of ischemia-reperfusion injury, the exact estrogen mediated signaling mechanisms have yet to be fully developed.

1.4.3 Estrogen and nitric oxide signaling

The junction between estrogen and NO cell signaling pathways appears to have a major role in the intrinsic cardioprotection that is exhibited in premenopausal women. Estrogen is known to induce expression and phosphorylation of eNOS in both vascular endothelial cells and cardiomyocytes. Expression and activation of eNOS by estrogen is believed to be mediated primarily through ER α in vascular endothelial cells and through ER β in cardiomyocytes (Cross et al. 2002; Lin et al. 2009b; Nuedling et al. 2001; Tan et al. 1999).

1.4.3.1 PI3K/Akt pathway

Preclinical studies have demonstrated cardioprotection with estrogen replacement in ovariectomized mice through the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (Figure 3) (Patten et al. 2004). The PI3K/Akt pathway is a component of the Reperfusion Injury Salvage Kinase (RISK) pathway. The RISK pathway encompasses a group of pro-survival protein kinases that when activated, provide protection in the setting of ischemia-reperfusion injury. The RISK pathway includes many other cardioprotective salvage kinases including Erk1/2, JNK, PKC, PKG, p70s6K, and GSK-3 β . Estrogen has been shown to increase the phosphorylation and activation of eNOS in the myocardium by binding to a membrane bound estrogen receptor which is believed to be coupled to PI3K (Guo et al. 2006; Hisamoto et al. 2001). Activated PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate phosphatidylinositol-3,4,5-bisphosphate (PIP₃). The membrane bound PIP₃ serves as a binding

site for Akt (also known as Protein Kinase B or PKB), which exists as one of three isoforms (Akt1, Akt2, and Akt3). Each isoform contains an N-terminal pleckstrin homology (PH) domain, a kinase domain, and a C-terminal regulatory domain containing a hydrophobic motif. Akt is normally kept in an inactive form through the intramolecular interaction between the PH and kinase domains. Binding of Akt to PIP₃ at the membrane induces a conformational change in Akt that enables PDK1 to phosphorylate Akt at the Thr308 site of the activation loop. Maximal Akt activity is achieved when the Ser473 site in the hydrophobic motif is phosphorylated by mTOR complex 2 (mTORC2). Activated Akt dissociates from the cell membrane and can phosphorylate a wide range of molecular targets involved in many different cellular process including metabolism, translation, proliferation, survival, and angiogenesis (Wiza et al. 2012). The serine 1177 site of eNOS is one target substrate of active Akt. Phosphorylated Akt (p-Akt) has been shown to be increased in female hearts during reperfusion and is believed to play an important role in sex-dependent cardioprotection (Bae and Zhang 2005; Shao et al. 2017b).

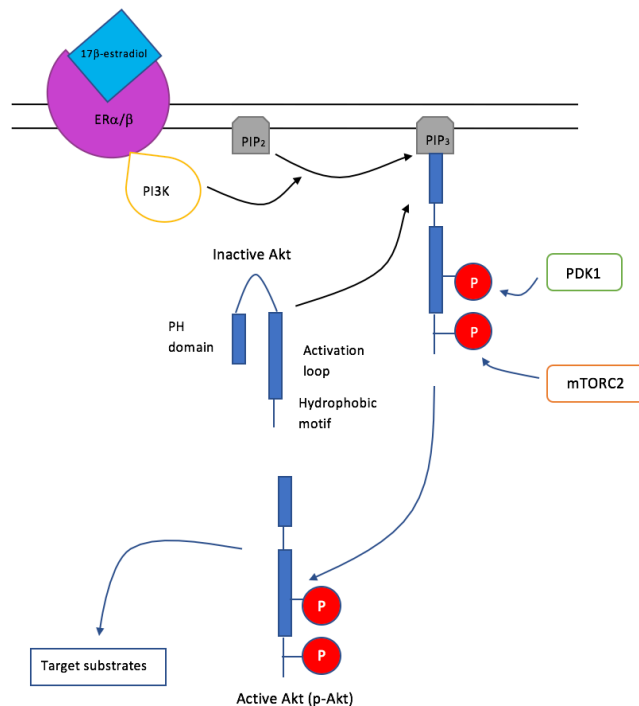


Figure 3. Estrogen - PI3K/Akt signaling pathway.

1.4.4 Clinical therapy

Our laboratory and others have identified intrinsic cardioprotective signaling mechanisms that serve to reduce cardiac injury in animal models of ischemia reperfusion-injury. Despite these advancements in cardiovascular research there has yet to be a cardioprotective therapy that has successfully reduced infarct size in humans, aside from reperfusion. While age and comorbidities are expected to partially underlie the failures of these clinical trials, environmental factors are expected to play a major role. Common exposures to environmental pollutants such as metals and fine particulate matter may abrogate the intrinsic cardioprotective signaling mechanisms, placing exposed populations at a heightened risk to develop cardiovascular disease. Arsenic is one common drinking water pollutant that is expected to contribute to the development of cardiovascular disease through the disruption of cardioprotective signaling mechanisms.

1.5 Arsenic toxicity

The toxic metalloid is a major water contaminant in the Western and Midwestern United States, and regions around the globe, including Chile, Bangladesh, India, and Taiwan. (Smith et al. 2000). Inorganic arsenic is a carcinogen and is toxic to most organ systems in the human body because it effects a variety of enzyme catalyzed biological reactions and cell signaling pathways (Miller et al. 2002). However, the cell specific mechanisms of toxicity are still not fully understood.

1.5.1 Arsenic within the environmental health paradigm

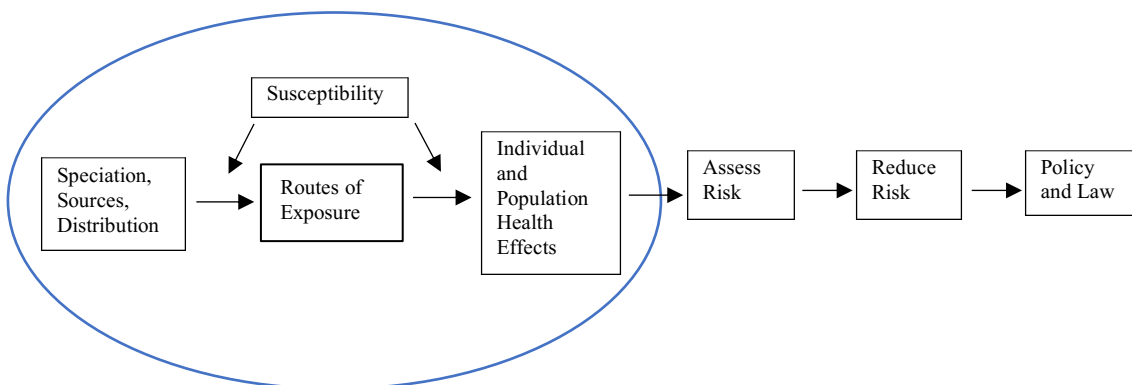


Figure 4. The environmental health paradigm.

1.5.1.1 Arsenic speciation, sources, and distribution

Arsenic is a naturally occurring metalloid element found in the soil, air, and water. The presence of arsenic in the environment has resulted from both natural and anthropogenic sources. The metalloid is a constituent of over 200 minerals. The element exists primarily in arsenates, sulfides, and sulfosalts, though it also exists in arsenides, arsenites, oxides, silicates, and as elemental arsenic (Garelick et al. 2008b). Arsenic can be integrated into a carbon and hydrogen structure in which it is referred to as organic arsenic. Arsenic can exist in an inorganic state, where the metalloid is combined with elements like oxygen and sulfur. In the inorganic form, arsenic exists in two different valence states that differ in composition and chemical reactivity. Arsenic in the trivalent state (As^{3+}) is referred to as arsenite and in the pentavalent state (As^{5+}) is referred to as arsenate. Arsenite and arsenate primarily exist in a complex with other elements including sodium, sulfur, and oxygen (Alamolhodaei et al. 2015). The toxicity of arsenic is mainly attributed to the inorganic species with trivalent arsenic being the most toxic. The mechanisms of toxicity are expected to differ between the two species of inorganic arsenic (Stea et al. 2014). Arsenic has become extensively mobilized as a result of industrial process.

Anthropogenic activities driving an increase in environmental arsenic concentrations includes the use of pesticides, the use of wood preservatives, the combustion of fossil fuels, mining, smelting, and the discharge of mining and ore processing waste. Mining and smelting serve as the most significant contributors to the environmental arsenic contamination. Arsenic is found in ores containing uranium, lead, gold, zinc, nickel, and cobalt. The mining operations generate tailings, residual waste rock from the mined ore, which contains a variety of arsenic compounds. The arsenic mineral forms regularly found on mining sites and of particular concern are arsenopyrite, arsenian, pyrite, realgar, and orpiment. The predominance of the different mineral forms and the mobility of the arsenic species is dependent on the soil type as well as the pH and redox potential of the soil. Closed mining sites lacking restoration programs leave behind tailings and waste materials that release arsenic into the environment as a result of natural weathering processes (Larios et al. 2012). Elevated concentrations of environmental arsenic can also result from burning arsenic rich coals. Coal burning for cooking and heating purposes results in arsenic contaminated consumer food products. Arsenic has been introduced into the environment as a result of extensive pesticide applications for agricultural activities. In addition to the usage of arsenic compounds as pesticides, chromated arsenicals have been used as wood preservatives within the timber industry (Garelick et al. 2008b).

1.5.1.2 Routes of exposure

Human exposure to arsenic occurs primarily through environmental and occupational sources (Alamolhodaei et al. 2015). Arsenic exposure can occur through inhalation, ingestion, or cutaneous absorption. Ingestion of drinking water serves as the greatest source of exposure to inorganic arsenic as the arsenic found in water is almost entirely inorganic. Due to the insolubility of elemental arsenic in water, the inorganic arsenic found in drinking water is in the form of arsenite and arsenate (Stea et al. 2014). Occupational exposures to arsenic are common in

chemical manufacturing, electronic manufacturing, and pesticide use and manufacturing workers. Humans can also become exposed to arsenic through medical treatments for disease states including psoriasis and acute promyelocytic leukemia (Alamolhodaei et al. 2015).

1.5.1.3 Individual and population health effects overview

Arsenic is a fascinating element as it has been considered to be both a poison and a medicine throughout history depending on the time and context. Arsenic's ability to induce apoptosis has allowed the metalloid to serve as a treatment of several diseases. Arsenic has been shown to induce complete remission in patients with acute promyelocytic leukemia, a cancer of the white blood cells (Soignet et al. 1998). While arsenic has been utilized as a medicine, the metalloid is primarily known as a driver of disease. Epidemiological studies have linked chronic and acute exposure to arsenic with a wide variety of disease states. Exposure to high doses of soluble inorganic arsenic may result in gastrointestinal, cardiopulmonary, cardiovascular, and central nervous system disorders. Intravenous infusion of trivalent arsenic for the treatment of promyelocytic leukemia has resulted in cases of sudden death. Human exposures to inorganic arsenic at fatally high doses present clinically with profound cardiac dysfunction, including QT prolongation, T-wave changes, ST segment depression, multifocal ventricular tachycardia and myocarditis (Alamolhodaei et al. 2015). Chronic arsenic exposure is believed to cause cancer, bronchopulmonary, cardiovascular, and metabolic diseases and neuropathies. Exposures to arsenic results in skin lesions and keratosis, the growth of keratin on the skin resulting in large and often benign skin growths (States et al. 2011). Chronic effects of arsenic on the cardiac system includes hypertension and the pathogenesis of atherosclerosis, peripheral vascular disease, and cardiomyopathy (Navas-Acien et al. 2005a). Due to the wide range of potential disease states resulting from arsenic exposure, arsenic likely has cell specific effects that dictate the clinical presentation.

1.5.2 Population based evidence of arsenic and cardiovascular disease

Epidemiological studies in areas of the world with high levels of arsenic in ground water have found associations with exposure to inorganic arsenic with risks of development of multiple forms of cardiovascular disease, including hypertension, carotid atherosclerosis, ischemic heart disease, and vascular disease mortality (Chen et al. 1995; Chen et al. 1996; Chen et al. 2007; Hsieh et al. 2011; Hsueh et al. 1998; Wang et al. 2002). Arsenic exposure through the ingestion of contaminated drinking water is a serious problem in the Asian nations of Taiwan, Bengal, and Bangladesh as a result of the naturally high geological arsenic concentrations. The situation in Bangladesh is being referred to as one of the largest mass poisonings of a population in history with the nation's drinking water being contaminated with inorganic arsenic concentrations up to 2500 ppb. It is estimated that 37 to 77 million people in the Bangladesh population are at risk of drinking the contaminated water (Smith et al. 2000). Epidemiological studies in Bangladesh have found strong associations between arsenic exposures, reduced methylation capacity, and risks of development of fatal and non-fatal forms of cardiovascular disease (Chen et al. 2013). Arsenic is considered the probable cause of blackfoot disease, which is a severe form of peripheral arterial disease endemic to southwestern Taiwan. Epidemiological studies in Taiwan also support a strong association between arsenic exposure and atherosclerosis. In Antofagasta, Chile, histopathological studies of children and young adults exposed to high levels of arsenic found thickening of arterial walls and early signs of myocardial infarction (Navas-Acien et al. 2005a). The effects of chronic exposures to low and moderate levels of arsenic were largely unknown until publication of the Strong Heart Cohort study shed light on the issue in 2013. Water contamination from abandoned hard rock mine lands has created cohorts of Native American communities facing exposure to low and moderate concentrations of arsenic in drinking water within the United States. Arsenic is the most common metal contaminant of the most extensively

mined metals which includes gold, uranium, copper, and lead. It is estimated that roughly 600,000 Native Americans live within 10 km of abandoned mine land (Lewis et al. 2017). The Strong Heart Study is the largest prospective cohort study in the United States to investigate the association between chronic exposure to low and moderate concentrations of inorganic arsenic and the incidence of cardiovascular disease. The study was conducted on three Native American communities in Arizona, Oklahoma, and North and South Dakota facing low to moderate concentration of arsenic exposure (<10 to 61 $\mu\text{g/L}$ in Arizona, <10 $\mu\text{g/L}$ in Oklahoma, and <1 to 21 $\mu\text{g/L}$ in the Dakotas). Using urine as a biomarker of exposure, Ana Navas-Acien's group found low to moderate chronic arsenic exposure to be associated with both fatal and non-fatal forms of cardiovascular disease, including coronary heart disease (Moon et al. 2013).

1.5.3 Arsenic metabolism

Most of the inorganic arsenic ingested is excreted rapidly in the urine. A relatively small amount of arsenic is modified by methylation and redox reactions to generate methylarsonate (MMA^{V}), methylarsonous acid (MMA^{III}), dimethylarsinic acid (DMA^{V}), and dimethylarsinous acid (DMA^{III}). The trivalent arsenic metabolites are believed to be more potent toxins than the pentavalent species (Miller et al. 2002). Reduced methylation capabilities, as a result of genetic polymorphisms in methylation enzymes, are associated with a greater incidence of cardiovascular disease resulting from arsenic exposure (Chen et al. 2013). The process of arsenic methylation is referred to as biotransformation. The overall structure of the pathway and the enzymes involved in arsenic metabolism are still debated. The generally accepted pathway of biotransformation occurring in the liver consists of a series of reduction and oxidation reactions, coupled with methylations (Figure 5). In the traditional pathway, pentavalent arsenic is formed before the trivalent arsenic species. There are still many uncertainties in this pathway and alternative

pathways have been proposed. A commonly cited alternative pathway of arsenic biotransformation that has gained significant support was proposed by Hayakawa (Figure 6) (Aposhian and Aposhian 2006).

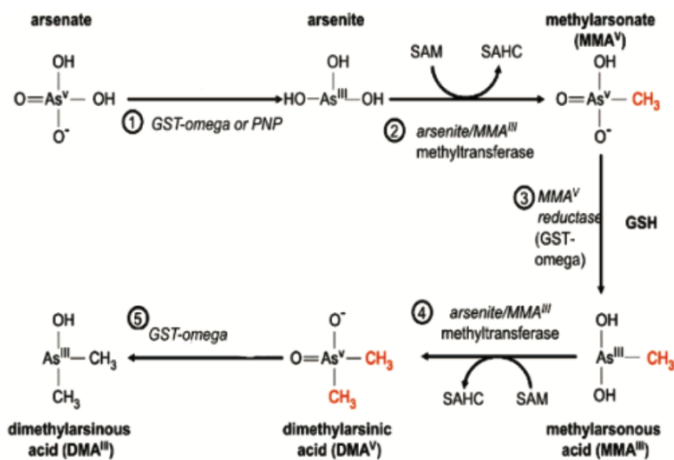


Figure 5. Traditional pathway of arsenic biotransformation credited to Challenger, Cullen, and Reimer (Aposhian and Aposhian 2006).

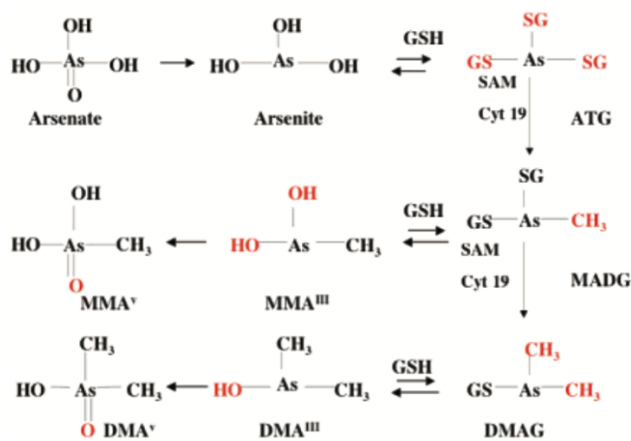


Figure 6. Alternative pathway of arsenic biotransformation proposed by Hayakawa (Aposhian and Aposhian 2006).

1.5.4 Arsenic's physiological modes of action

1.5.4.1 Protein interactions

Inorganic arsenic is considered a molecular analog of phosphate and can compete with phosphate in many biochemical reactions. By substituting for phosphate in oxidative phosphorylation, Inorganic arsenic inhibits ATP generation. Arsenite also has a high affinity for thiol groups and can bind to cysteine residues in proteins. Multiple studies have reported that arsenic exposure decreases GSH levels. Inorganic arsenic has also been shown to decrease the total accessible thiol content (Alamolhodaie et al. 2015). Inorganic arsenic binding to proteins can alter protein confirmation thereby disrupting protein functions and interactions with other proteins and DNA (Shen et al. 2013).

1.5.4.2 Generation of ROS

Arsenic trioxide is a known stimulator of ROS generation pathways (Han et al. 2010). Arsenic trioxide has been shown to induce the generation of intracellular ROS in a dose dependent manner (Chayapong et al. 2017). Arsenic also has been shown to increase the level of the oxidized form of GSH (GSSG) (Alamolhodaie et al. 2015). Cell cycle analysis of skin fibroblast cells treated with arsenic trioxide were observed to undergo cell cycle arrest, spending a significant amount of time in the G0/G1 phases with a reduction in the time spent in S phase. It is proposed that treatment with arsenic trioxide leads to oxidative stress, causing a shift in the cell cycle leading to DNA damage through a CHK-dependent signaling pathway (Chayapong et al. 2017).

1.5.4.3 Endocrine disruptor

Arsenic is a known disruptor of endocrine function and reproductive fitness (Shalat et al. 1996). Arsenic exposure has been directly linked to a later menarche age in females of Bangladesh villages with naturally high levels of arsenic in drinking water (Sen and Chaudhuri 2007). In addition to menarche age, arsenic exposure in Bangladesh has had a significant correlation with birth defects (von Ehrenstein et al. 2006). These studies provide evidence for arsenic driven dysregulation of estrogen levels in the body and an altered state of the hypothalamic-pituitary-gonadal axis, which plays a critical role in the development and regulation of many organ systems. It is proposed that arsenic disrupts endocrine and reproductive physiological function through the metalloids interaction with estrogen and estrogen signaling pathways. Arsenic has been shown to reduce plasma estrogen levels in a dose dependent manner (Chatterjee and Chatterji 2010a). It has been proposed that inorganic arsenic binds directly to 17 β -estradiol and abolishes the effects of the hormone (Kumar et al. 2016). Arsenite has been considered an environmental estrogen as it has been shown to bind to the hormone binding site of ER α blocking the site from estradiol activation (Miller et al. 2002).

1.6 Specific aims, objectives, and rationales

1.6.1 Investigate the effect of inorganic arsenic exposure on cardiac structure

The direct effects of arsenic exposure on cardiac structure is not fully understood. Arsenic exposure has been related to various cardiovascular diseases including hypertension. Long term hypertension and the associated increased afterload can lead to left ventricular hypertrophy which is indicated by an increase in left ventricular mass. Left ventricular hypertrophy is a strong

predictor of the cardiovascular and peripheral vascular complications, including ischemic heart disease, atherosclerosis, and stroke (Mehta et al. 2007; Verdecchia et al. 2007). Epidemiological studies have found a positive correlation between chronic arsenic exposure and cardiovascular diseases and risk factors including atherosclerosis, hypertension, diabetes, and peripheral arterial disease (Mazumder et al. 2012; Navas-Acien et al. 2005b; Newman et al. 2016; Rahman et al. 1999; Tseng et al. 2006). Female mice exposed to water containing 100ppb of sodium arsenite for 22 weeks were shown to exhibit increased systolic and diastolic blood pressure, indicating the manifestation of chronic hypertension. Echocardiographic analysis of the female mouse hearts was also indicative of concentric left ventricular hypertrophy (Sanchez-Soria et al. 2012a). We had reason to believe that 4 weeks of exposure to water containing sodium arsenite would lead to changes in the echocardiographic parameters indicating signs of left ventricular hypertrophy. We set out to determine if 4 weeks of arsenic exposure leads to cardiac remodeling in male and female mice using transthoracic echocardiography.

1.6.2 Determine the effect of inorganic arsenic exposure on sex-dependent susceptibility to ischemia-reperfusion injury

Consistent with previous animal studies, our group has observed greater post-ischemic functional recovery with female mouse hearts compared to males after 20 minutes of ischemia and 30 minutes of reperfusion (Figure 7) (Shao et al. 2016). An abolishment of female cardioprotection is observed in ovariectomized mice (Ross and Howlett 2012). The female heart's intrinsic resilience to ischemia-reperfusion injury is believed to be mitigated through estrogen signaling, as an abolishment of female cardioprotection is observed in ovariectomized mice (Ross and Howlett 2012). Estrogen has been observed to drive rapid, short term cardioprotective effects in the heart through the activation of signaling pathways (Menazza and Murphy 2016; Wu et al. 2011). One such cardioprotective effect of particular importance is the upregulation of eNOS and NO

signaling. Using SNO resin assistant capture (SNO-RAC) in tandem with mass spectrometry, we have observed that female hearts exhibited 65% more unique SNO protein identifications compared with male hearts (Figure 8) (Shao et al. 2016). This is suggestive of a protective role of nitric oxide signaling in the female heart. Trivalent arsenic is believed to disrupt endocrine function through the metalloid's interactions with estrogen signaling (Shalat et al. 1996). Arsenic exposures are associated with birth defects and a delayed onset of menarche (Chatterjee and Chatterji 2010a; Kumar et al. 2016; Sen and Chaudhuri 2007; Shalat et al. 1996; von Ehrenstein et al. 2006). Trivalent arsenic exposure has been shown to reduce serum estradiol levels in a dose and time dependent manner in female albino rats (Chatterjee and Chatterji 2010b). It is proposed that trivalent arsenic abrogates estrogen signaling through the direct binding to 17 β -estradiol (Kumar et al. 2016). We have reason to believe that arsenic may disrupt estrogen mediated sex-dependent cardioprotection. Therefore, we set out to investigate the effects of inorganic arsenic exposure on sex-dependent cardioprotection using the Langendorff-perfused mouse heart model of ischemia-reperfusion injury with male and female hearts. We hypothesized that trivalent arsenic treatment would abrogate sex-dependent cardioprotection through the metalloid's direct actions on 17 β -estradiol.

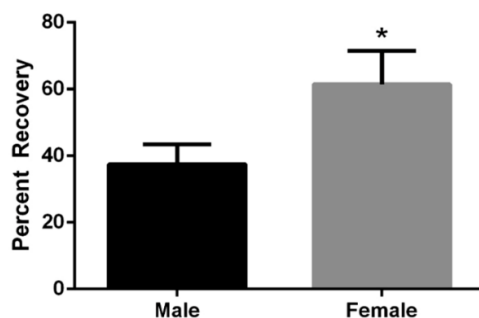


Figure 7. Female hearts exhibit greater post-ischemic functional recovery. Post-ischemic functional recovery in Langendorff-perfused male and female hearts following 20 min of ischemia and 30 min of reperfusion, * $p < 0.05$ vs. male (Shao et al. 2016).

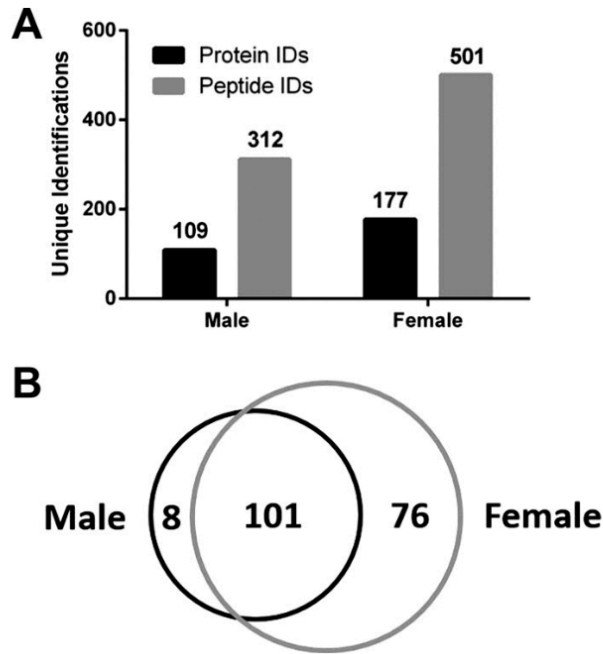


Figure 8. Female hearts show enhanced baseline myocardial SNO levels. (A) Total S-nitrosothiol (SNO) protein and peptide identifications at baseline in male and female hearts identified via SNO-resin-assisted-capture (SNO-RAC). (B) Venn Diagram depicting common and unique SNO protein identification in male and female hearts (Shao et al. 2016).

1.6.3 Investigate the effect of inorganic arsenic exposure on the PI3K/Akt signaling pathway

Ser1177 of eNOS is a downstream phosphorylation target of PI3K activated p-Akt.

Phosphorylation of eNOS at ser1117 leads to greater activity of eNOS and upregulation of NO signaling. Our group has observed increased expression and phosphorylation of eNOS at baseline in female hearts compared to males (Figure 9) (Shao et al. 2016; Shao et al. 2017a). Consistent with the increased expression and activation of eNOS observed in females, we have observed increased NO_x (NO, nitrite, nitrate) levels at baseline in whole heart homogenates of female hearts compared to male hearts (Figure 10). The addition of L-NIO, a specific inhibitor for eNOS,

led to a significant reduction in NO_x production. However, selective nNOS inhibition with SMLT had no effect on the NO_x levels, suggesting that eNOS expression and activation was responsible for the NO_x production. (Moon et al. 2013). Estrogen treatment has been shown to cause phosphorylation of Ser1177 and activation of eNOS in the heart through the PI3K/Akt pathway (Guo et al. 2006; Hisamoto et al. 2001). Vascular endothelial cells treated with sodium arsenite showed decreased protein levels of Akt1 and eNOS. The upregulation of eNOS was shown to attenuate the effects of the arsenite exposure (Tsou et al. 2005). An epidemiological study of an Inner Mongolia population chronically exposed to inorganic arsenic through drinking water were observed to have reduced serum concentrations of NO metabolites (Pi et al. 2000). We have reason to believe that trivalent arsenic is acting on Akt, eNOS, or on an upstream regulator like estrogen, causing a reduced activity of eNOS and reduced NO levels. We set out to investigate the effects of trivalent arsenic exposure on the PI3K/Akt pathway with a focus on eNOS activity in the setting of ischemia-reperfusion injury.

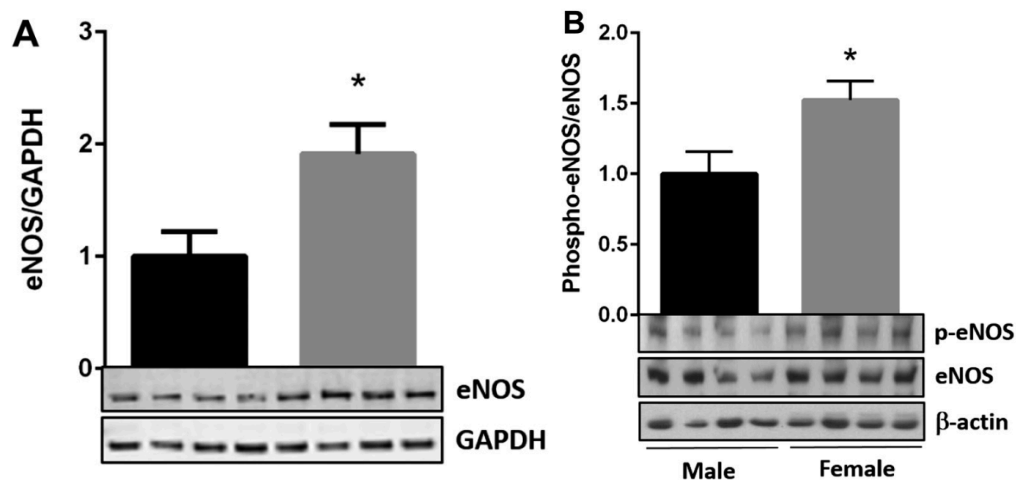


Figure 9. eNOS expression and phosphorylation is increased in female hearts. (A) Baseline eNOS expression and (B) phosphorylation at Ser1177 in male and female hearts as assessed via western blot. * $p < 0.05$ vs male (Shao et al. 2016).

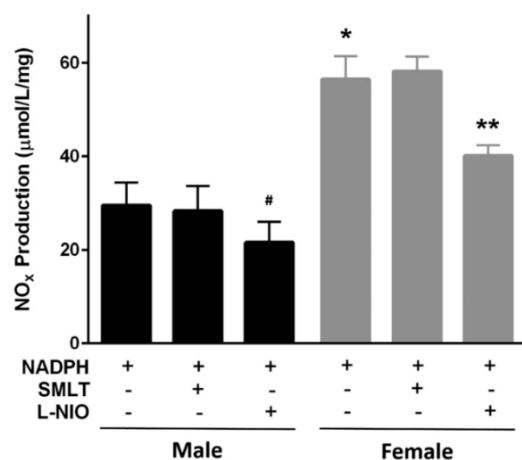


Figure 10. Baseline NO_x production is increased in female hearts. Baseline NO_x production assessed in male and female hearts via a colorimetric assay with SMLT serving as a selective inhibitor of NNOS and L-NIO as non-selective inhibitor of NOS. #p=0.075 vs. male +NAPDH - SMLT -LNIO, *p<0.05 vs. male +NAPDH -SMLT -LNIO, **p<0.05 vs female +NAPDH - SMLT -LNIO (Shao et al. 2016).

1.6.4 Overall study goal, hypothesis, and significance

The overall goal of the study is to characterize the effects of inorganic arsenic exposure on susceptibility to ischemia-reperfusion injury. Additionally, this project aims to identify the specific mechanisms through which inorganic arsenic exposure alters susceptibility to ischemia reperfusion injury. We hypothesized that iAS would inhibit estrogen signaling and receptor mediated gene expression thereby disrupting sex-dependent cardioprotection. The results of this study will provide valuable insights into the vast epidemiological evidence illustrating the positive correlation between arsenic exposure and ischemic heart disease. Identifying the mechanistic basis of inorganic arsenic cardiotoxicity may lead to better biomarkers of toxicity

and treatment options for chronic arsenic toxicity. We hope that our results highlight the immediate need for better arsenic contamination prevention and remediation plans. We will continue to work to establish firm mechanistic connections between environmental agents and cardiovascular disease.

CHAPTER 2

Inorganic Arsenic Exposure and Susceptibility to Ischemic Heart Injury

2.1 Introduction

Heart disease is the leading cause of morbidity and mortality, both in the United States and throughout the developed world (Benjamin et al. 2017; Roth et al. 2017). Epidemiological studies suggest that the majority of cardiovascular disease is preventable, highlighting the environmentally driven nature of the disease (Willett 2002). Compared to age-matched men, pre-menopausal women have historically had a reduced risk of cardiovascular disease. This sex-dependent phenomenon declines with menopause, suggesting a role for estrogen in the protection (Hayward et al. 2000). Females have been shown to exhibit endogenous cardioprotective signaling mechanisms derived from the beneficial effects of estradiol (Murphy et al. 2011; Shao et al. 2016). Several animal studies have shown estrogen and the activation of the estrogen receptors to be cardioprotective during ischemia-reperfusion injury (Booth et al. 2005; Hale et al. 1996; Lagranha et al. 2010; Lin et al. 2009a; M Wang et al. 2006). It is well established that females have a lower incidence of ischemic heart disease, left ventricle hypertrophy, and cardiac remodeling after an acute myocardial infarction. This is verified in preclinical animal studies in which females exhibit improved functional recovery and smaller infarct sizes following ischemia-reperfusion (I/R) (Bae and Zhang 2005; Shao et al. 2017a). Surprisingly, the divide between male and female ischemic heart disease mortality rates is narrowing according to the American Heart Association. Age-adjusted mortality after a myocardial infarction declined among males, but not females between 2001 to 2003 and 2007 to 2009 (Benjamin et al. 2017).

Numerous studies provide associations between heart disease and exposures to environmental pollutants, including particulate matter, gases, and metals (Cosselman et al. 2015). However, the exact mechanism(s) by which these pollutants drive heart disease have yet to be fully elucidated. These exposures may be differentially affecting males and females, effectively narrowing the gap in ischemic heart disease mortality rates. One pollutant directly linked to heart disease in epidemiological studies is the naturally occurring metalloid, inorganic arsenic (iAS). iAs toxicity is a global health problem, affecting millions of people lacking access to contaminant free drinking water. Chronic exposure to iAS has been linked to the manifestation of a vast array of disease states, including cancer (Lin et al. 2013; Smith et al. 2006), type 2 diabetes (Navas-Acien et al. 2008), neurological and cognitive defects (Wasserman et al. 2004), reproductive and developmental issues (Myers et al. 2010; Sen and Chaudhuri 2007; von Ehrenstein et al. 2006), and various cardiovascular diseases including atherosclerosis (Engel and Smith 1994; Huang et al. 2009), hypertension (Huang et al. 2007), and ischemic heart disease (Hsueh et al. 1998; Navas-Acien et al. 2005a; Tseng et al. 2003). The World Health Organization recommends that municipal water concentrations of iAS not exceed 10ppb. Despite this recommendation, the toxic metalloid remains at elevated levels and as a major water contaminant in the Western and Midwestern United States and regions around the globe including Chile, Bangladesh, India, and Taiwan (Nordstrom 2002). Elevated levels of iAS in water sources have resulted from the extensive mobilization of the species due to both natural and industrial processes including mining, agriculture, and fuel combustion (Garelick et al. 2008a; Larios et al. 2012). Relatively high ground water levels of iAS are found in Bangladesh, Taiwan, India, and Chile with populations exposed to water concentrations up to 2500 ppb (Nordstrom 2002). iAS water contamination is by no means restricted to developing regions of the world. A century of mining in the United States and the subsequent abandonment of mine lands has left Native American communities utilizing private drinking water wells exposed to toxic levels of iAS. The Strong Heart Study has illuminated the association between chronic exposure to low levels of iAS and

the incidence of both fatal and non-fatal forms of cardiovascular disease in Native American communities of Arizona, Oklahoma, and North and South Dakota (Moon et al. 2013).

Epidemiological studies link iAS exposure with risks of developing peripheral vascular disease, hypertension, carotid atherosclerosis, cardiomyopathy, and ischemic heart disease (Chen et al. 1995; Chen et al. 1996; Chen et al. 2007; Hsieh et al. 2011; Hsueh et al. 1998; Wang et al. 2002). iAS is believed to drive hypertension by promoting oxidative stress, lipid peroxidation, and loss of vasodilators in endothelial cells and smooth muscle cells (Ellinsworth 2015). Pressure overload due to hypertension is considered the major risk factor for the development of hypertrophic cardiomyopathy (Burchfield et al. 2013). The ability for iAs to induce cardiac remodeling either directly or indirectly by promoting peripheral vascular stress is still debated. Female mice exposed to 100 ppb iAS for 22 weeks were shown to have an increased left ventricular mass, interventricular septal wall thickness, blood pressure, and relative wall thickness illustrating some characteristics of concentric left ventricular hypertrophy (Sanchez-Soria et al. 2012b). Mouse models of atherosclerosis confirm that moderate iAs exposure enhances atherosclerotic plaque formation with less stable lesions through the promotion of pro-inflammatory conditions (Lemaire et al. 2011). Additionally, animal studies and In-vitro studies have shown that iAS exposure can disrupt cardiomyocyte calcium homeostasis, alter mitochondrial permeability, and cause cardiomyocyte apoptosis (Chen et al. 2010; Larochette et al. 1999; Raghu and Cherian 2009; Zhao et al. 2008).

The relationship between iAS exposure and ischemic heart disease is poorly understood, and mechanistic studies are lacking. Therefore, we set out to examine the effects of iAS exposure on susceptibility to ischemic heart injury using an ex vivo mouse model of injury in both males and females. Epidemiological studies provide a clear link between iAS exposure and ischemic heart disease. In addition to driving cardiovascular disease, iAS is considered an endocrine disruptor, altering sex steroid signaling and receptor mediated gene expression (Chatterjee and Chatterji

2010a; Davey et al. 2007; Watson and Yager 2007). Although female hearts exhibit an estrogen-dependent reduction in susceptibility to ischemic injury compared to males, females may be especially susceptible to iAS due to the endocrine disrupting effects. Nitric oxide signaling is at the root of estrogen-mediated cardioprotection. Our group and others have previously identified that female hearts exhibit higher nitric oxide levels and more unique S-nitrosylated peptides compared with male hearts (Shao et al. 2016). Endothelial nitric oxide synthase (eNOS) expression, phosphorylation, and activation is regulated by estrogen signaling and estrogen receptor mediated gene regulation (Deschamps et al. 2010; Marino et al. 2006). We have previously shown that female mice have higher levels of eNOS, and more activated eNOS compared to males (Shao et al. 2017a). Female albino rats exposed to 4000 ppb iAS were observed to have significantly diminished circulating estradiol, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels (Chatterjee and Chatterji 2010a). It has been proposed that iAS binds directly to 17 β -estradiol and abolishes the effects of the hormone (Kumar et al. 2016). Our results suggest that an environmental exposure such as iAS, can modulate susceptibility to ischemic heart disease. Our results further suggest that iAs can modulate myocardial nitric oxide signaling to either increase or decrease susceptibility to ischemic injury. It remains unclear if this result is mediated through changes in circulating estradiol. Future studies will aim to develop the connection between iAs endocrine disrupting effects, myocardial nitric oxide signaling, and alterations in susceptibility to ischemic heart injury. We hypothesize that iAS exposure alters the nitroso-redox equilibrium thereby differentially modulating susceptibility to ischemic injury in male and female hearts.

2.2 Materials and methods

2.2.1 Animals and iAS exposure protocol

Seven week old Male and female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). A total of 140 mice were used in this study. The mice were housed in a vivarium facility at Johns Hopkins University under specific pathogen-free conditions in rooms that maintain a constant temperature, humidity, and a 14-hour light/8-hour dark cycle. The animals were provided water and chow *ad libitum*. Each individual cage was provided with HEPA filtered air and bedding that was changed 2-3 times per week. The mice were provided Research Diet Inc. AIN-93G diet (Research Diet) and Nestle Pure Life water certified to contain no detectable level of iAS. The mice were acclimated to the feed, water, and housing conditions for one week prior to conducting the sodium arsenite exposure protocol. All mice utilized for the study were sexually mature at the age of eight weeks. The mice were given Nestle Pure Life drinking water containing 0 ppb (control), 10 ppb, 100 ppb, or 1000 ppb of iAS in the form of sodium arsenite (Sigma-Aldrich) for a total of four weeks of exposure. Water containing sodium arsenite were refreshed every 2-3 days to minimize oxidation. For all procedures, mice were anesthetized with a mixture of ketamine (Hofspira, Inc., Lake Forest, IL; 90 mg/kg) and xylazine (Sigma, St. Louis, MO; 10 mg/kg). After verifying adequate anesthesia via toe pinch, mice were subsequently euthanized via myocardial excision and exsanguination. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH publication NO. 85-23, revised 2011) and was approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

2.2.2 Assessment of cardiac remodeling with transthoracic echocardiography

Transthoracic echocardiography was performed in conscious mice using a Vevo 2100 system with a 40-MHz mechanical transducer (FUJIFILM VisualSonics, Toronto, Canada). The M-mode echocardiogram was acquired from the parasternal short axis view of the left ventricle (LV) at the level of the mid-papillary muscles and at a sweep speed of 200 mm/sec. From this axis view of the left ventricle, the following cardiac parameters were measured, including inter-ventricular septal thickness at end diastole (IVSD), LV chamber diameter at end diastole (LVEDD), posterior wall thickness at end diastole (PWTEd), and LV chamber diameter at end systole (LVESD). Using these parameters, the percent fractional shortening (FS %), relative wall thickness (RWT), and left ventricle mass (LV mass) were used in the estimation of cardiac contractility and left ventricle morphology. These indices were derived from the following equations:

$$FS (\%) = [(LVEDD - LVESD)/LVEDD] \times 100$$

$$EF (\%) = [(LVEDD^2 - LVESD^2)/LVEDD^2] \times 100$$

LV mass (mg): $1.055 [IVSD + LVEDD + PWTEd]^3 - (LVEDD)^3$, where 1.055 is the specific gravity of the myocardium

$$RWT = (PWTEd/LVEDD) \times 2$$

The end-diastolic and end-systolic ventricular volumes (EDV, ESV), stroke volume (SV), and the percent ejection fraction (EF) were estimated using the Simpson's method and the two chamber view of the heart on long axis.

2.2.3 Determination of susceptibility to ischemia-reperfusion injury

2.2.3.1 Langendorff perfused heart model

The isolated perfused heart model, developed by Oscar Langendorff in 1897, was used to simulate ischemia-reperfusion injury. Despite its development over 100 years ago, the Langendorff-perfused heart model remains as the standard tool for investigating cardiac injury. The model has served invaluable in cardiac research allowing for the development of an understanding of basic heart physiology such as myocardial infarction, electrophysiology, coronary vascular function, and clinically presenting cardiac disease states (Bell et al. 2011). With the development of more advanced genomics, proteomics, and metabolomics techniques, the Langendorff-perfused heart model has allowed for the investigation of complex myocardial physiology and cellular signaling pathways. Limitations of the isolated heart model includes the absences of normal hormonal and neuronal regulation of cardiac function. Additionally, the model is characterized by high coronary flow and edema as the perfusate generally lacks a cellular component. The isolation of the heart from confounding peripheral influences can serve as a strength of the model as it allows for a better understanding of the direct actions of toxins and pharmaceuticals (Skrzypiec-Spring et al. 2007). The ex-vivo approach allows for the isolation of multiple experimental variables that can influence contractile function including temperature, oxygen, and calcium ion concentrations of the perfusate. Anoxia and hypoxia can easily be imposed on the heart providing a valuable tool of simulating both local and global ischemia (Skrzypiec-Spring et al. 2007). The Langendorff heart model utilizes a retrograde perfusion through the aorta with fluid flow that is opposite to the normal physiological flow. Due to the retrograde flow of the perfusate the leaflets of the aortic arch close and the fluid enters the coronary arteries directly via the ostia at the aortic root. Through the ostia and the right and left branching coronary arteries, the ventricular mass is

perfused and the fluid empties into the right atria through the coronary sinus (Sutherland and Hearse 2000).

2.2.3.2 Krebs-Henseleit perfusate

Krebs-Henseleit buffer (KHB) was used as the perfusate in the Langendorff-perfused heart assay. This is the most common bicarbonate buffer utilized by researchers investing cardiac pathology with a Langendorff-perfused heart model (Bell et al. 2011). The KHB solute concentrations were set at 120 mmol/L NaCl, 4.7 mmol/L KCL, 1.2 mmol/L KH_2PO_4 , 25 mmol/L NaHCO_3 , 1.2 mmol/L MgSO_4 , 11 mmol/L D-glucose, 1.75 mmol/L CaCl_2 . To mimic physiological conditions, the pH of the KHB was set to 7.4 and the temperature was maintained at 37 °C.

2.2.3.3 Ischemia-reperfusion protocol, myocardial infarct staining, and functional recovery analysis

After conducting the post exposure transthoracic echocardiography measurements, the mice were subjected to the ex-vivo Langendorff-perfused heart assay. Mice were anesthetized with a mixture of ketamine and xylazine via an intraperitoneal injection. Heparin was then administered as an anticoagulant. Hearts were excised from the male and female mice and placed directly in ice-cold Krebs-Henseleit buffer. The aorta of the heart was cannulated and perfused in a retrograde fashion in the dark at a constant pressure of 100 cm of buffer with the KHB at pH 7.4 and a temperature of 37 °C. For both the control and the treatment groups, the hearts were subjected to a standard ischemia-reperfusion protocol of 20 minutes of equilibration, 20 minutes of ischemia, and 120 minutes of reperfusion (Figure 11). A latex balloon connected to a pressure transducer was inserted into the left ventricle to measure left ventricular developed pressure

(LVDP) and heart rate. Both left ventricular developed pressure and heart rate were monitored and recorded using the PowerLab system. The rate pressure product was used as a measure of cardiac contractile function.



Figure 11. Ischemia-reperfusion protocol.

After the completion of the ischemia-reperfusion protocol, hearts were perfused with Triphenyl tetrazolium chloride (TTC) and fixed in formalin. Triphenyl tetrazolium chloride was used as a gross histochemical staining technique to facilitate the rapid identification of the myocardial infarction at the macroscopic level. The Triphenyl tetrazolium chloride interacts with intact dehydrogenase enzymes to form a red precipitate. Necrotic tissue lacks the presence of active dehydrogenases and therefore fails to form a red precipitate (Fishbein et al. 1981). White Infarcted tissue can easily be distinguished from the viable tissue stained red. In addition to assessing myocardial infarct size, functional recovery as determined following the completion of the ischemia-reperfusion protocol. The post-ischemic functional recovery will be determined by assessing left ventricular developed pressure and heart rate during the myocardial perfusion to be able to calculate the rate pressure product for measurement of cardiac contractile function. The postischemic functional recovery is expressed as a percentage of the preischemic rate pressure product.

2.2.4 Liver alanine transaminase activity

Alanine transaminase (ALT) activity was assessed in whole liver homogenates from male and female mice, previously exposed to 0 ppb iAS (control) or 1000 ppb iAS for four weeks via drinking water, using a commercially available kit (Abcam). Approximately 15 mg of liver tissue was homogenized in 200ul of assay buffer for each sample using a Dounce glass homogenizer on ice and centrifuged at 14,000 g for 10 minutes at 4 °C to pellet debris. The supernatant was recovered as total crude homogenate. Sample protein concentration was determined using the Bradford protein assay. Exactly 5 µl of sample was added to 15 µl of assay buffer in an individual well of a 96-well plate. To initiate the reaction, 100ul of a mixture containing the ALT substrates alanine and α-ketoglutarate, ALT enzyme cofactors, and an oxired probe was added to each individual well. ALT catalyzes the transfer of an amino group from alanine to α-ketoglutarate to produce pyruvate and glutamate. Pyruvate then converts the colorless probe to a color with a 570 nm wavelength. Pyruvate production was thereby indirectly monitored via absorbance at 570 nm wavelength for 10 minutes at 37°C. ALT activity was calculated from pyruvate produced between reaction time 2 minutes and 10 minutes using the equation below.

$$\text{ALT activity} = ((B/\Delta T * V) * DF) / [S]$$

ΔT = reaction time

B = nmol pyruvate produced over ΔT

V = volume of sample added

DF = sample dilution factor

[S] = sample protein concentration

ALT activity was expressed in terms of Units where 1 Unit (U) of ALT is the amount of ALT which generates 1.0 μ mol of pyruvate per minute at 37°C. Samples were ran in duplicate and average ALT activity was determined.

2.2.5 Plasma 17 β -estradiol levels

Blood was extracted from the inferior vena cava and collected in tubes treated with heparin. Cells were removed by centrifugation at 14,000 g for 10 minutes at 4°C. Supernatant was collected and stored at -80°C. Plasma samples were analyzed for 17 β -estradiol concentrations at the UVA Center for Research in Reproduction Ligand Assay and Analysis Core using a commercially available estradiol Elisa kit (Calbiotech Inc) per the manufacturers instruction.

2.2.6 Whole heart homogenate preparation

A separate set of mice were given Nestle Pure Life drinking water containing 0ppb (control) or 1000ppb of iAS in the form of sodium arsenite for a total of four weeks of exposure. Hearts were excised and Langendorff perfused for 20 minutes in the dark. Following perfusion, hearts were snap frozen in liquid nitrogen. All subsequent procedure were completed in the dark. Hearts were powdered on liquid nitrogen with a mortar and pestle, and resuspended in 1.0 mL of homogenization buffer containing (in mmol/L): sucrose (300), HEPES-NaOH pH 8.0 (250), EDTA (1), neocuproine (0.1), and Triton-X 100 (0.5%). An EDTA-free protease inhibitor tablet (Roche, Indianapolis, IN) was added just before use. Samples were then homogenized using a Dounce glass homogenizer on ice and centrifuged at 14,000 g for 30 minutes to pellet debris. The supernatant was recovered as total crude homogenate.

Protein concentration was determined using the Bradford protein assay. Total homogenates were then aliquoted and stored at -80°C.

2.2.7 Western blots for eNOS, p-eNOS, Akt, p-Akt, nNOS, and iNOS

Samples were separated on a 4-12% Bis-Tris SDS-PAGE gel and transferred to a nitrocellulose membrane (Life Technologies). Membranes were blocked with 5% (w/v) nonfat dried milk in Tris-buffered saline with 0.1% Tween-20 for one hour, and subsequently incubated with primary antibodies against phospho-Akt Ser473 (1:1000, Cell Signaling Technology, Danvers, MA, 4060S), total Akt (1:1000, Cell Signaling Technologies, 4691S), phospho-eNOS Ser1177 (1:500; Cell Signaling Technology, 9570s), total eNOS (1:250; Santa Cruz Biotechnology, Dallas, TX), nNOS (1:250, Santa Cruz Biotechnology, Dallas, TX), or iNOS (1:250, Santa Cruz Biotechnology, Dallas, TX). Membranes were then probed with the corresponding secondary antibodies for 1 hour and visualized by electrogenerated chemiluminescence (Life Technologies). Densitometry was assessed using ImageJ software (National Institutes of Health, Bethesda, MD).

2.2.8 Statistics

Results are expressed as the mean \pm SEM. Statistical significance ($p < 0.05$) was determined between groups using a Student's t-test for two groups or a two-way ANOVA with Tukey's multiple comparison correction for multiple groups.

2.3 Results

2.3.1 iAS has sex disparate effects on susceptibility to ischemic heart injury

To determine the effect of iAS exposure on sex-dependent susceptibility to ischemic heart injury, we subjected male and female mouse hearts, previously exposed to 0 ppb (control), 10 ppb, 100 ppb, or 1000 ppb for four weeks, to a standard I/R protocol of 20 mins of equilibration, 20 mins of ischemia, and 120 mins of reperfusion, and assessed recovery of function and myocardial infarct size. In mice exposed to 10ppb or 100ppb of iAs, we detected no significant changes in susceptibility to I/R injury in both male and female groups; post-ischemic functional recovery and infarct size was not significantly different compared to non-iAS exposed mice. Following 1000ppb iAS exposure, however, we identified substantial sex-dependent changes in I/R susceptibility. iAS-treated female hearts showed a significant decrease in post-ischemic functional recovery and increased infarct size, while iAS-treated males showed significantly enhanced post-ischemic functional recovery and reduced infarct size (Figure 12).

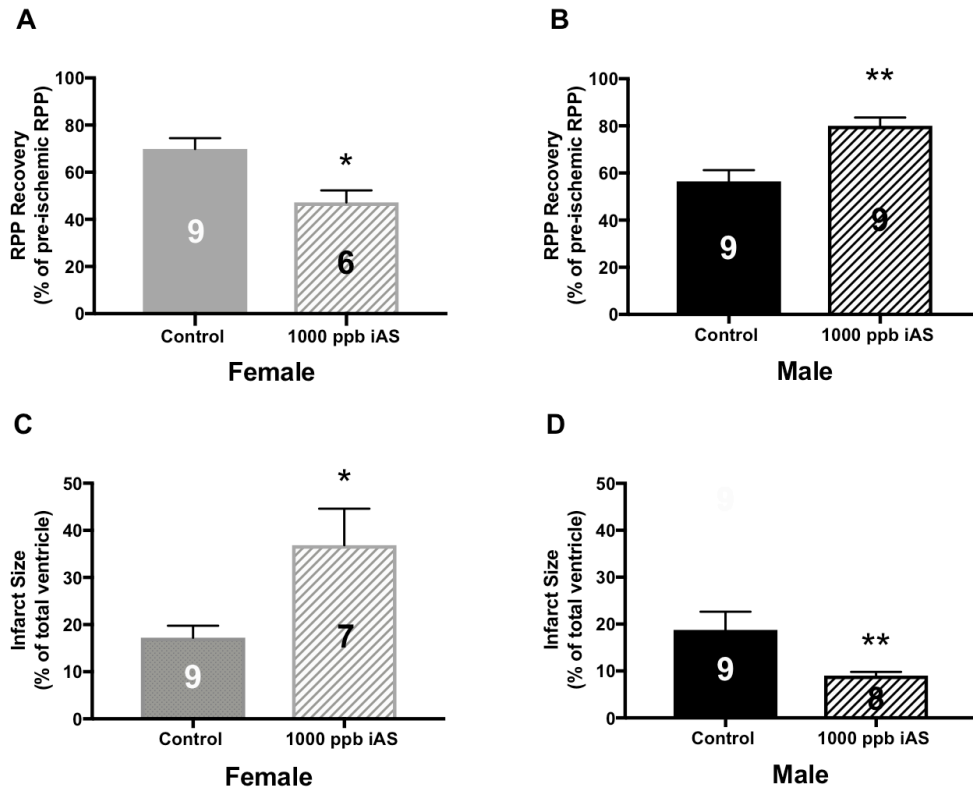


Figure 12. Four weeks of exposure to 1000 ppb iAS results in sex disparate effects on susceptibility to ischemic heart injury. After four weeks of exposure to 0 ppb (control) or 1000 ppb iAS via drinking water, hearts were excised and Langendorff-perfused. For all treatment groups, hearts were equilibrated for 20 mins, subjected to 20 mins of ischemia, and reperfused for 120 mins. (A-B) Post-ischemic functional recovery at 90 mins of reperfusion. (C-D) Post-I/R myocardial infarct size. Hearts were perfused with 10% Triphenyl tetrazolium chloride (TTC) and fixed in formalin following reperfusion. ImageJ software was used to analyze infarct size, expressed as the percentage of total area of cross-sectional slices. Numbers inside each bar represent the number of mice used for each group, * $p < 0.05$ vs. control female; ** $p < 0.05$ vs. control male.

2.3.2 No difference in weight change, feed consumption, or water intake is observed with iAS exposure

Since dietary restriction and dehydration modulates susceptibility to ischemia-reperfusion injury (Mitchell et al. 2010), we monitored feed and water intake during the acclimation to housing conditions and throughout the duration of the exposure. Feed and water consumption was recorded when replaced every 2-3 days. Mouse weight was measured once a week. We observed no significant difference between treatment groups in feed consumption or water intake during acclimation to the housing conditions or during the exposure (Figure 13). Additionally, we did not observe a significant difference in overall weight change or rate of weight change during the exposure protocol at any of the three iAS doses (Figure 14).

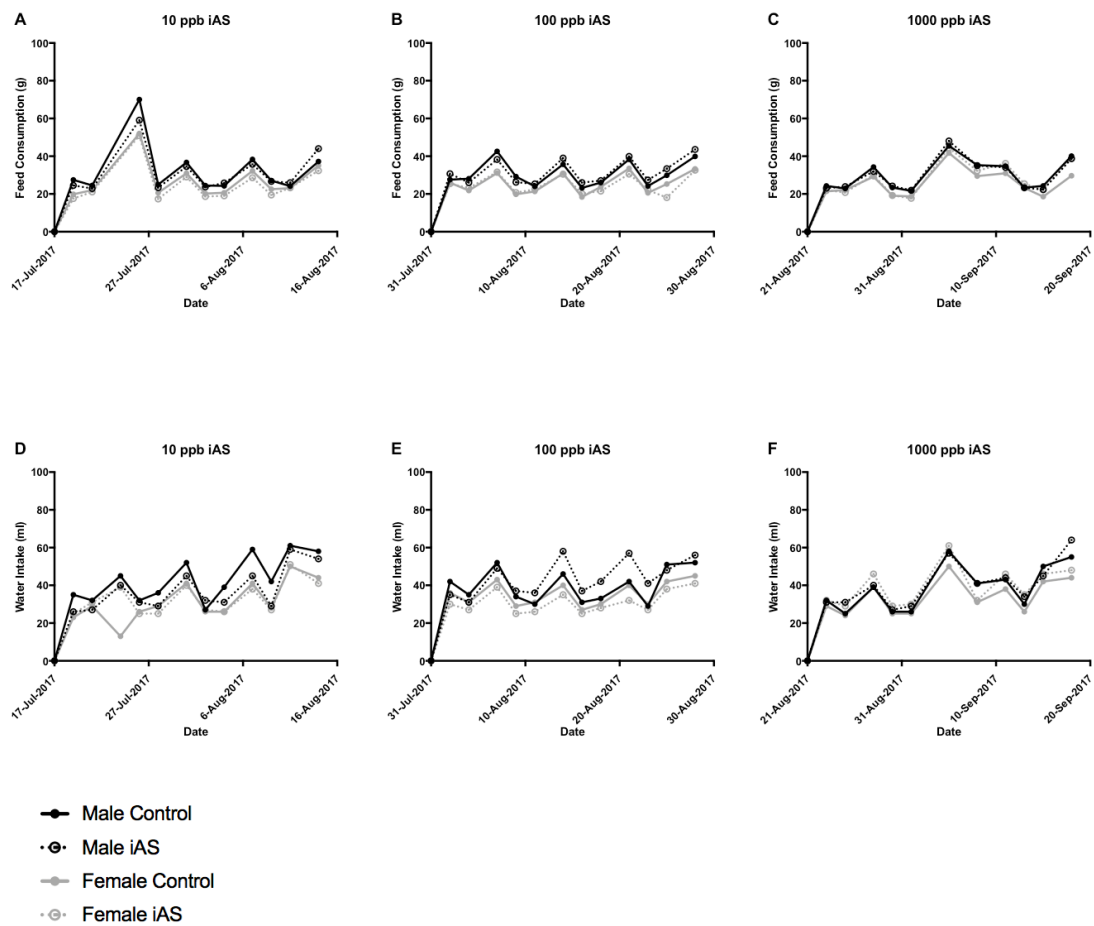


Figure 13. No difference in feed consumption or water intake is observed with iAS exposure. (A-C) Feed consumption during exposure protocol for all three iAS doses (n= 5 per group). (D-F) Water intake during exposure protocol for all three iAS doses (n = 5 per group).

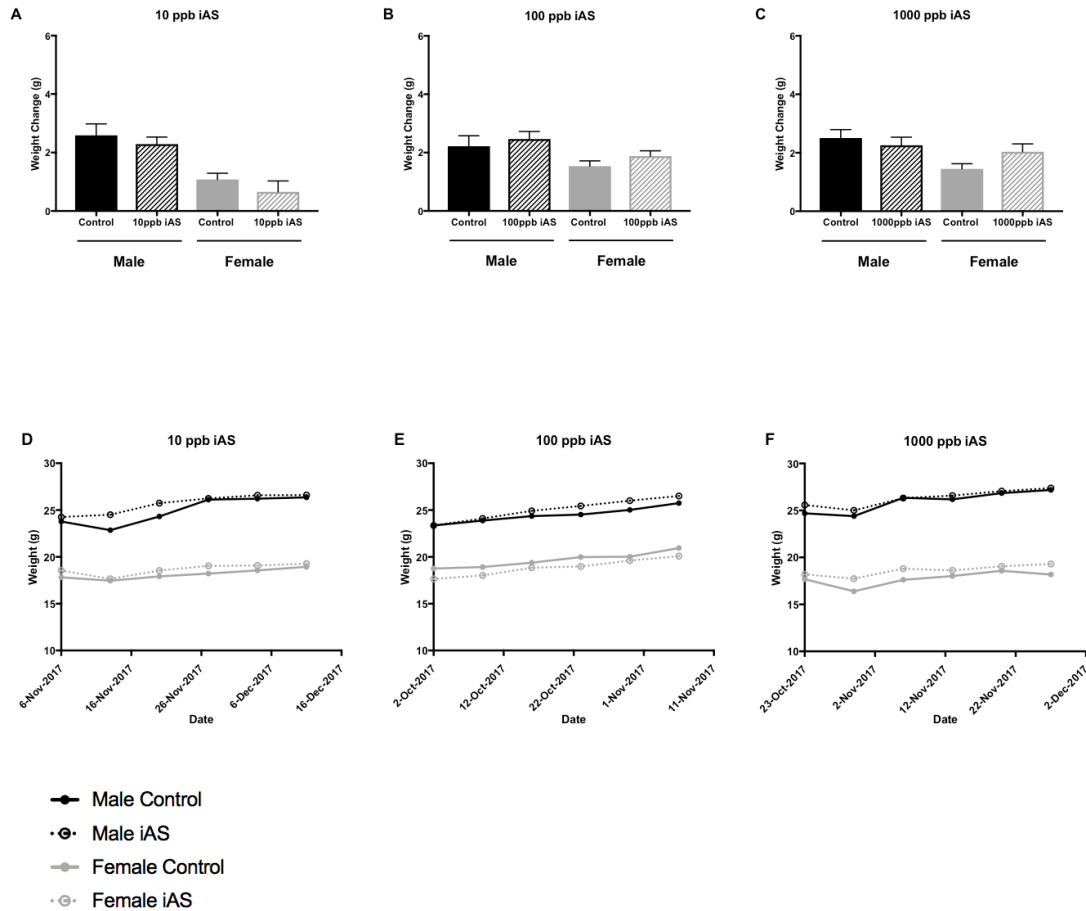


Figure 14. No difference in overall weight change or rate of weight change is observed with iAS exposure. (A-C) Overall weight change expressed as the difference between the post-exposure weight and the pre-exposure weight (n = 10 per group). (D-F) Mouse weight recorded weekly during the duration of the iAS exposure (n = 5 per group).

2.3.3 Moderate iAS exposure induces modest cardiac remodeling with no change in gross cardiac function in females

iAS exposure is associated with major risk factors for pathological cardiac hypertrophy, including hypertension, atherosclerosis, and type two diabetes. Since pathological cardiac hypertrophy increases susceptibility to ischemic heart injury (Penna et al. 2011), we assessed the cardiac

geometry and function of both male and female hearts, pre- and post-exposure, for all three iAS doses using transthoracic echocardiography. We did not observe significant changes in cardiac geometry or function with exposure to the 10 ppb or 100 ppb iAS doses in male or female mice (data not shown). We did observe a modest but significant increase in IVSd and LV mass in 1000 ppb iAS-treated females (vs. non-treated females) (Table 1). Despite observing a significant increase in IVSd and LV mass, we did not observe changes in myocardial function (i.e., ejection fraction, fractional shortening, heart rate, etc.) that would signify cardiac dysfunction and/or pathological cardiac hypertrophy.

Table 1. Echocardiographic parameters for pre- and post-1000 ppb iAS exposure.

Echo Parameter	Male Control		Male 1000 ppb iAS exposure		Female Control		Female 1000 ppb iAS exposure	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
HR, bpm	739.00 ±19.07	703.00 ±19.40	697.00 ±10.44	714.00 ±21.30	696.00 ±14.00	703.00 ±12.90	697.00 ±19.40	725.00 ±18.97
EF, %	77.83 ±0.99	76.40 ±0.85	75.60 ±0.78	71.76 ±2.90	77.14 ±0.94	71.11 ±0.39	77.39 ±0.58	72.84 ±2.44
FS, %	58.43 ±1.08	53.15 ±1.29	56.98 ±0.75	54.26 ±2.68	58.34 ±1.62	55.28 ±0.45	60.55 ±0.50	54.23 ±3.05
EDV, µl	21.17 ±1.47	22.38 ±2.54	19.93 ±3.10	22.41 ±1.46	20.61 ±2.30	16.65 ±1.22	15.40 ±1.88	18.50 ±1.25
ESV, µl	4.71 ±0.42	5.23 ±0.51	4.78 ±0.62	6.22 ±0.51	4.10 ±0.16	4.81 ±0.34	3.45 ±0.34	5.03 ±0.53
SV, µl	16.46 ±1.12	17.15 ±2.06	15.15 ±2.49	16.19 ±1.60	15.87 ±1.68	11.84 ±0.89	11.95 ±1.55	13.48 ±1.00
IVSd, mm	1.01 ±0.04	1.12 ±0.04	1.06 ±0.04	1.08 ±0.04	0.97 ±0.03	0.98 ±0.01	0.94 ±0.03	1.10 ±0.02*
LVIDd, mm	2.72 ±0.09	2.84 ±0.08	2.67 ±0.06	2.85 ±0.06	2.51 ±0.07	2.50 ±0.06	2.55 ±0.05	2.55 ±0.06
LVIDs, mm	1.13 ±0.05	1.33 ±0.03	1.15 ±0.02	1.31 ±0.10	1.02 ±0.04	1.12 ±0.04	1.01 ±0.01	1.16 ±0.07
LVPWd, mm	0.99 ±0.02	1.00 ±0.04	1.01 ±0.04	1.06 ±0.04	0.90 ±0.02	0.93 ±0.03	0.94 ±0.03	1.02 ±0.03
RWT	0.73 ±0.02	0.71 ±0.05	0.76 ±0.03	0.74 ±0.03	0.72 ±0.03	0.74 ±0.03	0.74 ±0.02	0.80 ±0.04
LV Mass, mg	90.31 ±5.54	104.68 ±4.83	93.35 ±7.76	107.37 ±7.34	71.99 ±3.05	73.66 ±2.72	74.71 ±4.71	89.76 ±2.23*

* $p < 0.05$ vs. post-exposure female control (n = 4 - 5 per group)

HR: heart rate; EF: ejection fraction; FS: fractional shortening; EDV: end-diastolic ventricular volume; ESV: end-systolic ventricular volume; SV: stroke volume; IVSd: interventricular septal thickness at end diastole; LVIDd: left ventricular internal diameter at end diastole; LVIDs: left ventricular internal diameter at end systole; LVPWd: left ventricular posterior wall at end diastole; RWT: relative wall thickness; LV Mass: left ventricle mass.

2.3.4 Liver toxicity was not observed with iAS exposure

The remaining experiments were conducted on control mice and mice exposed to 1000 ppb iAS as we observed cardiac phenotypic changes only at this concentration in our four week exposure model. To investigate if the change in susceptibility to ischemic injury was a result of toxicity to other organ systems, we examined the liver, which is a major target of both arsenic toxicity and arsenic carcinogenesis. Noncirrhotic fibrosis is common in individuals chronically exposed to a high dose of iAS. ALT activity reflects damage to hepatocytes and is considered a specific and highly sensitive biomarker of hepatotoxicity (Aubrecht et al. 2013). Evaluation of ALT activity revealed no signs of toxicity in either male or female mice treated with 1000 ppb iAs (Figure 15).

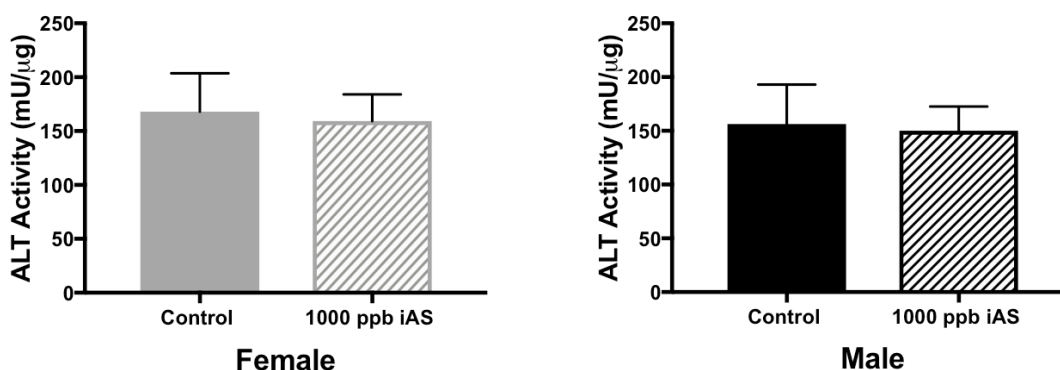


Figure 15. Liver toxicity is not observed in mouse iAS exposure model in male or female mice according to ALT activity, an established clinical biomarker of liver toxicity. ALT activity was assessed in whole liver homogenates of male and female mice previously exposed to 0 ppb (control) or 1000 ppb iAS for four weeks. ALT activity was expressed in terms of Units where 1 Unit (U) of ALT is the amount of ALT which generates 1.0 μmol of pyruvate per minute at 37°C. (n = 10 per group).

2.3.5 iAS exposure reduces circulating 17 β -estradiol levels in females

Since female hearts normally exhibit an estrogen-dependent reduction in susceptibility to ischemic injury and we have observed significantly greater susceptibility to I/R injury with iAS exposure, we examined the plasma 17 β -estradiol levels of 1000 ppb iAS-treated male and female mice. We observed a decrease in 17 β -estradiol levels in iAS-treated females (vs. non-treated females), but this decrease was not significant. No change was observed in iAS-treated males (Figure 16).

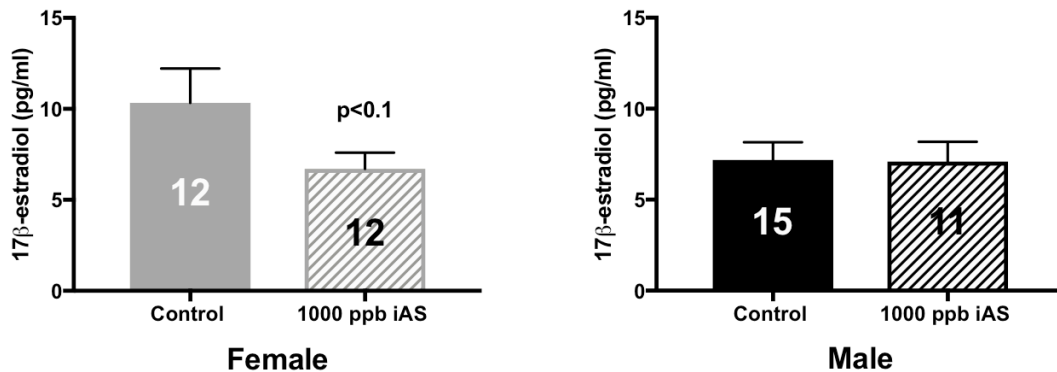


Figure 16. iAS exposure reduces circulating 17 β -estradiol levels in females, but this decrease is not significant. 17 β -estradiol levels were assessed by ELISA in plasma samples of male and female mice previously exposed to 0 ppb (control) or 1000 ppb iAS for four weeks. Numbers inside each bar represent the number of mice used for each group.

2.3.6 iAS alters myocardial nitric oxide signaling independent of PI3K/Akt signaling pathway

NO signaling appears to be a key component of sex-dependent cardioprotection, and we have shown that female hearts exhibit increased expression and phosphorylation of eNOS, as well as enhanced phospho-Akt levels, at baseline compared to males (Shao et al. 2016; Shao et al.

2017a). Therefore, we next examined Akt and eNOS as potential targets of iAS exposure. eNOS protein levels were significantly decreased in whole heart homogenates from both iAS-treated male and female hearts (Figure 17G-H). eNOS phosphorylation at Ser1177 was also significantly elevated in iAs-treated male hearts and remained unchanged in iAS-treated female hearts (Figure 17E-F). No change was observed in either Akt or phospho-Akt levels in iAs-treated male or female hearts (Figure 17A-D).

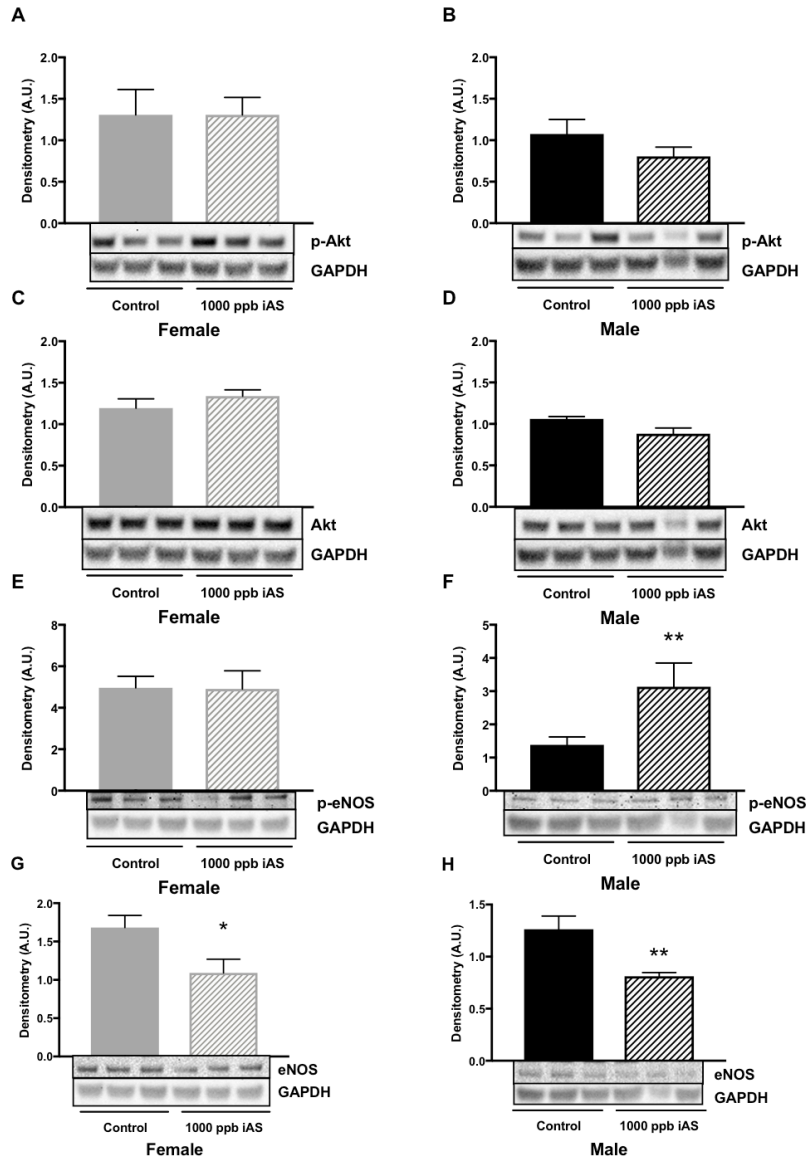


Figure 17. iAS alters myocardial nitric oxide signaling independent of PI3K/Akt signaling pathway. (A-B) Western blot depicting phosphorylated (Ser473) Akt levels in control and 1000 ppb iAS-treated male and female hearts. (C-D) Western blot depicting Akt levels in control and 1000 ppb iAS-treated male and female hearts. (E-F) Western blot depicting phosphorylated (Ser1177) eNOS levels in control and 1000 ppb iAS-treated male and female hearts. (G-H) Western blot depicting eNOS levels in control and 1000 ppb iAS-treated male and female hearts (n = 4 - 5 per group; 2 technical replicates per mouse). *p<0.05 vs. control female; **p<0.05 vs. control male.

2.3.7 iAS does not alter nNOS or iNOS expression in the heart

To further investigate the effect of iAS exposure on NO signaling, we probed for the other two other isoforms of NOS, nNOS and iNOS. We observed no significant change in nNOS protein levels (Figure 18A-B), and iNOS expression was not detected in either male or female hearts (Figure 18C). Macrophage cell lysate was used as a positive control for iNOS.

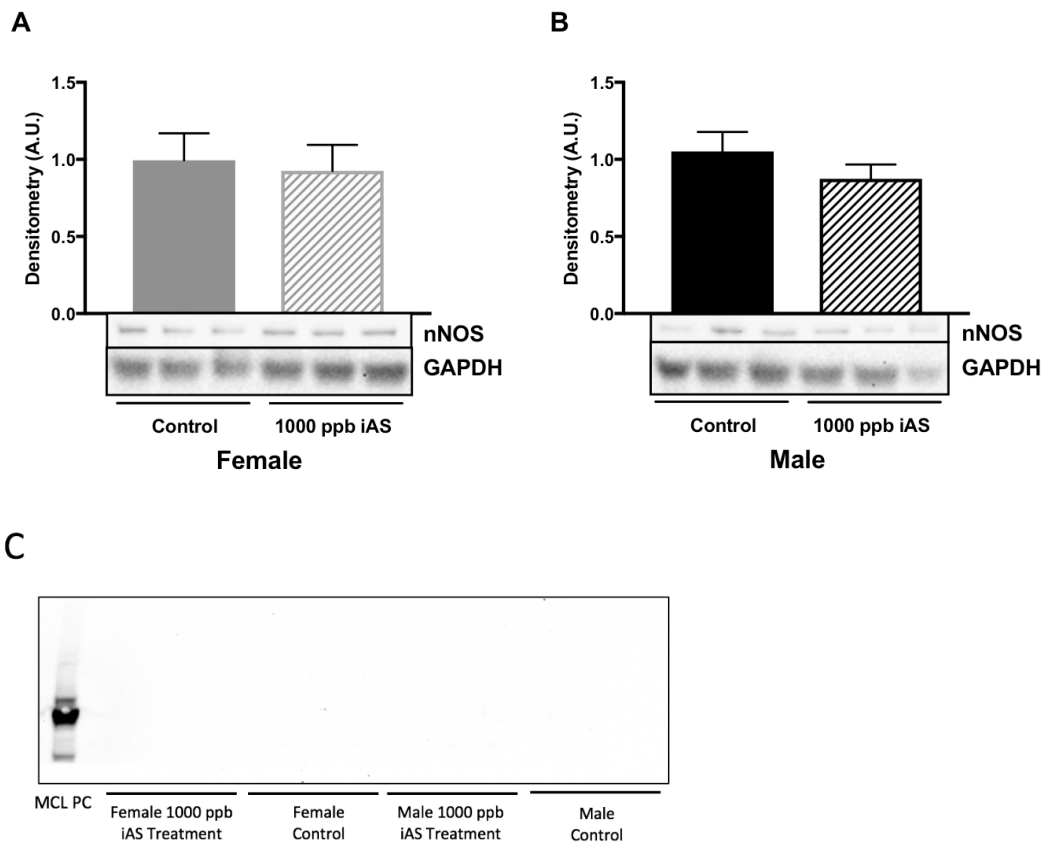


Figure 18. iAS does not alter nNOS or iNOS expression in the heart. (A-B) Western blot depicting nNOS levels in control and 1000 ppb iAS-treated male and female hearts. (C) Western blot depicting iNOS levels in control and 1000 ppb iAS-treated male and female hearts. Macrophage cell lysate (MCL) was used as a positive control for iNOS (n = 5 per group).

2.4 Discussion

Numerous epidemiological studies link iAS exposure to an increased risk of ischemic heart disease. Despite the clear epidemiological link to ischemic heart disease, the mechanism(s) by which arsenic alters susceptibility to ischemic heart injury is unclear. We hypothesized that females are especially susceptible to ischemic injury with iAS exposure due to endocrine disrupting effects. We have demonstrated for the first time in an animal model that iAS exposure alters susceptibility to ischemic heart injury. We identified substantial sex-dependent changes in I/R susceptibility following only four weeks of 1000 ppb iAs exposure. As hypothesized, iAS-treated female hearts exhibited significantly more injury following I/R with a significant decrease in post-ischemic functional recovery and increased infarct size. Surprisingly, a four week 1000 ppb iAS treatment appears to protect the male heart against ischemic heart injury. iAS-treated male hearts showed significantly enhanced post-ischemic functional recovery and reduced infarct size following I/R.

After establishing the sex disparate effect of iAS exposure on susceptibility to ischemic heart injury, we proceeded to investigate the underlying mechanistic basis. First, we considered the link between iAS exposure and peripheral vascular diseases, including atherosclerosis and hypertension, that may cause pathological cardiac hypertrophy and place the heart at a greater susceptibility to I/R injury. An epidemiological study of children in Mexico has linked early life iAS exposure to elevated blood pressure, higher LV mass, and reduced EF (Osorio-Yanez et al. 2015). Transthoracic echocardiography revealed only modest changes in cardiac geometry with a significantly increased IVSd and LV mass in only 1000 ppb iAS-treated female mice. These results are consistent with a previous study that identified an increase in IVSd and LV mass in addition to a significant increase in RWT in female mice exposed to 100 ppb iAS for 22 weeks (Sanchez-Soria et al. 2012b). Despite the modest changes in cardiac geometry, we did not

observe any changes in function that would place the heart at a greater susceptibility to ischemic heart injury.

With pathological cardiac hypertrophy excluded as a culprit for the altered susceptibility to ischemic heart injury, we considered toxicity to other organ systems as a potential mechanism. We examined toxicity of the liver, a major target of both arsenic toxicity and arsenic carcinogenesis, using liver ALT activity as a biomarker of hepatocyte damage. Elevated ALT activity has been reported in mouse models exposed to 3200 ppb iAS for 12 months and 4900 ppb iAS for 10 months (Santra et al. 2000; Tan et al. 2011). Consistent with previous animal studies utilizing similar arsenic exposure models in rats and C57BL/6J mice (Arteel et al. 2008; Lemaire et al. 2011), we did not observe altered ALT liver enzyme activity in either male or female mice treated with 1000 ppb iAS for four weeks (Figure 15).

Next, we hypothesized that iAS decreased circulating 17β -estradiol levels in females, thereby disrupting estrogen signaling and receptor mediated gene expression, leading to a greater susceptibility to I/R injury. iAS has been shown to reduce estrogen levels in a dose dependent manner as well as disrupt estrogen signaling and receptor mediated gene expression in vitro and in female albino rats (Chatterjee and Chatterji 2010a; Davey et al. 2007; Watson and Yager 2007). We observed reduced circulating 17β -estradiol levels in iAS-treated females (vs. non-treated females), but this was not statistically significant. Additionally, 17β -estradiol levels were unexpectedly low across all treatment groups and we did not detect a difference between male and female mice. More sensitive and specific methodology will need to be developed to examine 17β -estradiol levels in mouse models. Nonetheless, we have previously shown eNOS and Akt to be involved in sex-dependent cardioprotection so we proceeded to examine those targets. We have shown that eNOS protein levels are significantly decreased in whole heart homogenates

from both iAS-treated male and female hearts. We have also shown that eNOS phosphorylation (Ser1177) levels are significantly elevated in iAs-treated male hearts and unchanged in iAs-treated females. This increase in activation of eNOS may be driving the protection observed in iAS-treated males. Additionally, we have shown that the other two NOS isoforms, iNOS and nNOS, remain unchanged with iAS exposure. Altogether, our results suggests that iAS exposure differentially effects NO signaling in male and female hearts thereby driving sex-dependent changes in susceptibility to ischemic heart injury.

In summary, we have shown that an environmental exposure can modulate susceptibility to ischemic heart injury. We demonstrated that iAS exposure has sex-dependent effects on susceptibility to ischemic heart injury. Our results suggest that environmentally relevant concentrations of iAS may be of much greater health risk to women.

2.5 Future directions

Future studies will aim to further illuminate the mechanism(s) underlying the altered susceptibility to ischemic injury observed in male and female hearts exposed to 1000 ppb iAS for four weeks. Our results suggest that iAS exposure differentially effects NO signaling in male and female hearts thereby driving sex-dependent changes in susceptibility to ischemic heart injury. Therefore, a main focus of future studies will be on the effect of iAS exposure on myocardial nitric oxide signaling and the nitroso-redox equilibrium.

We have identified significantly reduced eNOS levels in both iAS-treated male and female hearts. We will determine if the decreased eNOS protein levels is a result of changes in gene regulation, an issue of translation, or a result of increased eNOS degradation. We will use quantitative PCR

to examine the relative eNOS mRNA expression in male and female hearts exposed to 0 ppb (control) and 1000 ppb iAS for four weeks.

We have also identified significantly elevated phosphorylated (Ser1177) eNOS levels in iAS-treated male hearts despite observing no change in PI3K/Akt signaling. The Ca²⁺/calmodulin-dependent protein kinase (CaMKII) has previously been shown to phosphorylate Ser1177 of eNOS (Fleming et al. 2001). CaMKII activity is regulated by cytoplasmic calcium levels and previous in-vitro studies have shown that iAS can increase intracellular calcium levels (Florea et al. 2005). iAS may be increasing phosphorylated (Ser1177) eNOS levels either by increasing CaMKII expression or by increasing CaMKII activity by increasing intracellular calcium levels. We will examine CaMKII phosphorylation and expression by western blot.

While we have identified significant and differential changes in eNOS expression and activation in iAS-treated male and female hearts, we cannot conclude that NO signaling is enhanced in male hearts and reduced in female hearts. We will examine NO_x (nitrite, nitrate) levels in iAS-treated male and female hearts to understand how NO levels correlate with eNOS expression and activation. We will then examine the effect of iAS exposure on NO signaling in male and female hearts by looking at SNO peptide levels using the SNO resin assistant capture (SNO-RAC) in tandem with mass spectrometry.

In addition to investigating changes in NO signaling, we will look at ROS levels in male and female hearts previously exposed to 0 ppb (control) and 1000 ppb iAS for four weeks. iAS has been shown to stimulate ROS generation pathways and oxidative stress (Han et al. 2010). We will examine hydrogen peroxide levels post-iAS exposure in male and female hearts using the Amplex Red Hydrogen Peroxide Assay kit.

We have observed modest but significant changes in gross cardiac structure with no change in cardiac function in female mice exposed to 1000 ppb iAS for four weeks. We believe that with prolonged iAS exposure, female mice will exhibit pathological hypertrophy with changes in cardiac function in addition to structure. We also expect to see change in male cardiac structure with prolonged exposure to iAS. A future study will examine gross cardiac structure and function changes in male and female mouse hearts following treatment with 0 ppb (control), 10 ppb, 100 ppb, and 1000 ppb iAS for different durations of exposure (16, 24 , 32 wks). We will also monitor tail blood pressure periodically throughout the IAS exposure protocol to determine if changes in cardiac structure and function have resulted from hypertension. We will continue to examine changes in susceptibility to ischemic injury using the Langendorff perfused heart assay as we expect that we will observe changes in susceptibility at much lower concentrations of iAS with longer exposure times. Additionally, we expect that males will exhibit greater susceptibility to ischemic heart injury at more prolonged exposure times. We believe that any protective signaling in males that was upregulated with the brief iAS exposure will become pathological or overwhelmed by toxicity in the prolonged iAS exposure model.

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Biography

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